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To cite this version:
Cécile Echalier, Laurine Valot, Jean Martinez, Ahmad Mehdi, Gilles Subra. Chemical cross-linking methods for cell encapsulation in hydrogels. Materials Today Communications, Elsevier, 2019, 20, pp.100536. 10.1016/j.mtcomm.2019.05.012. hal-02151576

HAL Id: hal-02151576
https://hal.archives-ouvertes.fr/hal-02151576
Submitted on 18 Dec 2019

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Chemical cross-linking methods for cell encapsulation in hydrogels

Cécile Echalier¹,², Laurine Valot¹,², Jean Martinez¹, Ahmad Mehdi² and Gilles Subra¹

¹IBMM, Univ Montpellier, CNRS, ENSCM, Montpellier, France.
²ICGM, Univ Montpellier, CNRS, ENSCM, Montpellier, France.

Cell-encapsulating hydrogels are of tremendous interest in regenerative medicine. Tissue engineering relies on biomaterials able to act as artificial extracellular matrices to guide cells towards the development of new tissues. Therefore, considerable efforts have been made to design biomaterials which mimic cells’ native environment, thus encouraging natural behavior. The choice of biomaterial in which cells are embedded is crucial for their survival, proliferation and differentiation. Being more stable, chemical hydrogels are preferred over physical hydrogels as cell-laden substrates. When designing chemical hydrogels, scientists must choose not only the nature of the network (synthetic and/or bio-polymers) but also the type of cross-link bridging hydrogel constituents. For that purpose, numerous chemistries have been used (i) to introduce reactive functions on the hydrogel precursors and (ii) to form covalent bonds in the presence of living cells. The review will discuss the advantages and limitations of each strategy.

Cell-laden hydrogels are of paramount importance in tissue engineering. This area of research aims to produce living tissues to be used as tissue grafts to repair or replace damaged biological tissues/organs.[1,2] For this purpose, cells are seeded in a scaffold that will support them and provide signals to guide them towards the development of a new functional tissue. Ultimately, the engineered tissue will be transplanted into the patient where it is expected to restore biological tissue function. Alternatively, cells can be encapsulated in injectable hydrogels [3–6] to achieve cell delivery at the site of interest for in situ regeneration of a tissue. The hydrogel serves as temporary scaffold which provides mechanical support and appropriate signals until new tissue is formed by the injected cells. In both cases, the choice of the biomaterial in which cells are embedded is crucial for tissue development. This material must provide the cells with an environment which is as similar as possible to their native environment.

1.1. Hydrogels as mimics of the ECM
In biological tissues, cells are surrounded by a complex and bioactive extracellular matrix (ECM).[7–10] An interstitial fluid is entrapped in a network constituted of hydrated proteoglycans coiled around fibrous proteins such as collagen, fibronectin, elastin and laminin (Fig. 1). Collagen is the most abundant fibrous protein in the ECM, its fibers providing tensile strength to the surrounding tissue. Together, all these components confer a hydrogel-like consistency to the ECM.
The role of the ECM is not only to provide mechanical support to the cells but also to regulate their behavior. Indeed, ECM is involved in cell survival, proliferation, migration and differentiation. This matrix holds cells together in tissues and maintains cells in a buffered aqueous environment. It enables the diffusion of oxygen, nutrients and metabolic products. It sequesters and delivers growth factors and other biological signaling molecules while controlling the diffusion of cell-secreted substances. In addition, it provides cell-matrix interactions. For instance, fibronectin introduces cell adhesion sites to attachment-dependent cells. Mechanical signals from ECM also affect cell fate. Matrix rigidity greatly influences cell differentiation. Finally, ECM is highly dynamic, constantly undergoing remodeling by proteases and matrix metalloproteinases (MMPs) which allows cell migration and proliferation. Considering the importance of ECM to so many cellular processes, it is a challenge to design artificial matrices that faithfully replicate the structure and functions of native ECM.

Given their high water content (typically $w_{\text{water}} \gg w_{\text{polymer}}$, with $w$ the mass fraction), hydrogels have been investigated as artificial ECM.[12–15] They are made of hydrophilic polymers cross-linked into water-insoluble polymeric networks. Their elastic properties provide a physical environment close to native soft tissues. Their mechanical properties can be tuned by modifying the degree of interaction (i.e. the non-covalent interactions or covalent cross-linking) between the polymer chains. However, although a high cross-linking density leads to high mechanical strength, it also results in lower swelling capabilities and decreased mesh sizes, which has an impact on biomolecule diffusion and cell migration. Therefore, a subtle balance should be struck between the physical properties of the hydrogel.

1.2. Hydrogel precursors
It seems obvious to use natural biopolymers as hydrogel network precursors and many hydrogels used for cell encapsulation are prepared from natural polymers, like collagen, hyaluronic acid (HA), alginate, chitosan, dextran and fibrin.[16,17]

Nonetheless, biopolymers suffer several drawbacks. They can be expensive to produce and often ill-defined. Their properties may vary from batch to batch and are difficult to tune. In addition, they could present a risk of contamination with pathogenic substances and they might be immunogenic. In contrast, synthetic polymers such as polyethylene glycol (PEG) and poly(vinyl alcohol) (PVA) are easy to produce on a large scale and are well-defined. Their chemical modification is simple and resulting hydrogels are highly reproducible. However, they are bio-inert, in the sense that they are biocompatible but cannot promote cell behavior. The term permissive hydrogels is used for them, as opposed to promoting hydrogels made of natural polymers.[20] Nevertheless, it is possible to confer some bioactivity to permissive hydrogels by functionalizing them.[35] The modification of artificial matrices with cell adhesive peptides is very common. Peptide sequences are often derived from ECM proteins.[36] Among them, the fibronectin-derived Arg-Gly-Asp (RGD) sequence which is a recognition motif for cell integrin receptors is the most frequently used.[37,38] Other peptide sequences of interest have proven to be useful in designing artificial ECM. Anseth and co-workers demonstrated that KLER sequence could promote cartilage-specific matrix deposition and organization by binding and stabilizing collagen.[39] Growth factors have also been introduced in artificial matrices. For instance, Phelps et al. engineered a system in which vascular endothelial growth factor (VEGF) was sequestrated within a hydrogel and delivered to the cells upon proteolytic degradation of the gel.[13] Tsurkan et al. described a PEG-heparin hydrogel in which heparin serves as reservoir for growth factors.[40]

1.3. Physical and chemical hydrogels

Depending on the type of interactions involved in the formation of the matrix, two types of hydrogels can be defined: physical and chemical hydrogels. Physical hydrogels result from the formation of a physical network involving weak interactions such as ionic interactions, hydrogen bonds, \(\pi-\pi\) stacking, Van der Waals forces and hydrophobic interactions.[18] Physical hydrogels are mostly obtained from biopolymers [16,17] assembled through multiple noncovalent interactions whose weakness is counterbalanced by their large number. Alginate in particular has been extensively investigated as ECM substitute due to its ease of gelation driven by complexation of calcium cations.[19] Alternately, small synthetic molecules, called hydrogelators, may self-assemble in water to form three-dimensional supramolecular networks.[20] Self-assembling peptides hold a prime position among hydrogelators [21–26]][27]. One of the main advantages of physical hydrogels is that no reactive functional group is required for cross-linking and establishment of the tridimensional network. Thus, the synthesis of precursors is straightforward (no further modification with reactive groups is required) and cross-reactions in vivo are avoided. However, physical hydrogels often suffer from poor mechanical properties owing to the weak interactions involved in their formation. While shear-thinning behavior is an asset for hydrogel injection, it prevents applications, such as articular cartilage repair, in which the hydrogel will be subjected to shear forces. Overall, the mechanical properties of physical hydrogels are difficult to tune since it is impossible to play with the cross-linking density. The concentration in precursors can be increased to get stiffer gels but it may create large osmotic gradients in vivo. It is also difficult to control the degradability of physical hydrogels. When associations between the molecules are formed by weak individual interactions, the local concentration can be reduced over time by dilution with physiological fluids, which leads to loss of structural integrity. On the other hand, chemical hydrogels are cross-linked through covalent chemical bonds, providing improved mechanical properties and stability to the resulting hydrogels compared to physical hydrogels. This makes
them more suitable for long-lasting tissue engineering applications. A wide range of precursors, displaying reactive functions, is available to prepare chemical hydrogels via different methods. The second part of the review will discuss the advantages and limitations of each strategy.

Mechanical properties of hydrogels (e.g. rheological behavior, swelling) are governed by the nature of the (bio)polymer, its size and concentration, and by the cross-linking density. Another way of tuning properties of a chemical hydrogel is to prepare an interpenetrating network by adding another (bio)polymer forming a physical hydrogel into the cross-linked one.[28,29] It is worth noting that the nature of the chemical cross-linkage itself will have a limited impact on the mechanical properties.

However, the degradation of the network which is mainly dependent on the nature of the (bio)polymer, can also be tuned by the stability of the cross-linkages. This is an important feature as it allows cell growth and migration, leading to the replacement of an artificial matrix by natural tissue.[35,41] Hydrolysable bonds (e.g. esters) can be introduced in the network providing a time-dependent degradation.[42–44] More interestingly, cell-responsive hydrogels can be obtained with enzyme-sensitive peptide cross-linkers, ensuring that the gel degradation will match cell growth. Patterson and Hubell reported hydrogels sensitive to matrix metalloproteinases (MMPs).[45,46] This review focuses on the different chemical reactions that can be used to cross-link cell-laden hydrogels. As already mentioned, the choice of chemistry has little influence on the mechanical properties of the resulting materials. Therefore, the mechanical properties of the hydrogels will not be discussed herein. However, we will highlight the cleavage of the bonds leading to the degradation of the network.

2. Strategies for the synthesis of chemical hydrogels for cell encapsulation

2.1. Cell encapsulation: implications for hydrogel cross-linking methods and elementary bricks

Herein, we will discuss cross-linking methods leading to a covalent hydrogel compatible with live cell encapsulation. The main parameters for cell-friendly embedment are summarized below.

- Avoidance of organic solvents
- Gelation in an aqueous solution isotonic to cytosol
- Maintenance of pH between 7.2 and 7.5
- Phosphate-buffered saline and, better yet, cell culture media (recommended)
- Temperature maintained at between room temperature and 40°C. Ideally, the in vitro gelation process should be performed at 37°C in a humidified 5% CO₂ atmosphere.[30]

These requirements have a strong impact on the hydrogel precursors and the cross-linking chemistry. First of all, cross-linking must proceed in aqueous conditions. Therefore, hydrogel precursors should be water-soluble. For a homogeneous cell distribution within the hydrogel, the precursor solution must transform into a solid hydrogel relatively quickly to avoid sedimentation of the cells after their introduction. Precursors, catalysts, initiators, cofactors, additional reagents, etc. must be cytocompatible and the cross-linking reaction should not generate cytotoxic by-products. This is one of the reasons why macromolecular precursors are often preferred, since most of them are less cytotoxic than small molecules. The cytotoxicity is often related to a lack of selectivity of the reactive functions borne by the precursors. For example, aldehydes and epoxides may react with biomolecules from live cells, thus interfering with native biological processes. Indeed small bifunctional molecules such as
epichlorohydrin,[31] glyoxal[32] and 1,4-butanediol diglycidyl ether[33] have been used to cross-link polymers, but presented some cytotoxicity because of their lack of selectivity. The ‘bio-orthogonality’, which regarding materials is the selectivity of precursors for network formation versus reaction with unwilling natural biomolecules, is crucial for the design of convenient hydrogels.[34] Therefore, we will report herein only bio-orthogonal cross-linking strategies (Table 1). Recent reviews summarizing advances in cross-linking chemistries are available;[35,36] these are focused on commonly used PEG-based hydrogels.

Table 1. Examples of cross-linking reactions used to prepare cell-encapsulating hydrogels
<table>
<thead>
<tr>
<th>Cross-linking reaction</th>
<th>Commercially available reagents for the functionalization of hydrogel precursors</th>
<th>Synthetic polymers and/or biopolymers used</th>
<th>Encapsulated cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chain-growth photopolymerization</strong></td>
<td>Acryloyl chloride, methacryloyl chloride, methacrylic anhydride, 2-isocyanato-ethylmethacrylate, glycidyl methacrylate</td>
<td>PEG[37–39], PLA-b-PEG-b-PLA[4], PVA[4], chondroitin sulfate[4], alginate[40], hyaluronic acid[41–43], collagen[44,45], chitosan[46,47], gelatin[48–50]</td>
<td>Aortic smooth muscle cells[37,44], calvaria osteoblasts[38], pancreatic[39], articular chondrocytes[40,46,47,51,52][86][86][86][86][86][85], valve interstitial cells[41], induced pluripotent stem cells neural progenitor cells[42]</td>
</tr>
<tr>
<td><strong>Step-growth photopolymerization</strong></td>
<td>5-norbornene 2-carboxylic acid, cysteine derivatives incorporated through peptide chemistry, dithiothreitol, 3-mercaptopropionic acid</td>
<td>PEG[53–55], gelatin[56]</td>
<td>Mesenchymal stem cells[53,54,56], embryonic stem motor neurons[55]</td>
</tr>
<tr>
<td><strong>Michael addition</strong></td>
<td>N-(2-aminoethyl)maleimide trifluoroacetate salt, 4-mercaptophenylpropionic acid, mercaptoisobutyric acid, 2-dimethyl-3-(4-mercaptophenyl)propionic acid</td>
<td>PEG[57,58], heparin[59]</td>
<td>Pancreatic islets[57], myoblasts[58]</td>
</tr>
<tr>
<td><strong>Strain-promoted azide alkyne cycloaddition</strong></td>
<td>2-(2-cyclooctyn-1-yloxy)-acetic acid bicyclo[6.1.0]non-4-yn-9-yl-methanol or methyl N-succinimidyl carbonate, 11,12-didehydro-5,6-dihydro- dibenzo[a,e]cycloocten-5-ol, 11,12-didehydro-γ-oxo-dibenzo[b,f]azocine-5(6H)-butanoic acid, sodium azide, 4-azidobutanoic acid</td>
<td>PEG[60–69]</td>
<td>3T3 fibroblasts[61,63,64], embryonic stem cell derived motor neurons[65], mesenchymal stem cells[66,68], bone marrow stromal cells[67], HeLa cells[69]</td>
</tr>
<tr>
<td><strong>Diels-Alder reaction</strong></td>
<td>Furfuryl methacrylate, furfurylamine, 3-(2-Furyl)propanoic acid, N-(2-aminoethyl)maleimide TFA salt, 4-(4-N-maleimidophenyl) butyric acid hydrazide, N-maleoyl-β-alanine, N-methoxycarbonylmaleimide</td>
<td>PEG[70–75], hyaluronic acid[70,72,73,76], dextran[77], poly(N,N-dimethylacrylamide-co-furfuryl methacrylate)[71],</td>
<td>Chondrogenic cells[70]</td>
</tr>
<tr>
<td>Reaction Type</td>
<td>Compound Details</td>
<td>Related Cells/Composites</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Inverse electron demand Diels Alder reaction</td>
<td>$5{4{1,2,4,5$-tetrazin-3-yl}benzylamino$}5$-oxopentanoic acid, 5$\text{-norbornene \text{2-carboxylic acid}}$</td>
<td>Mesenchymal stem cells$[78,81]$, prostate cancer cells$[80]$, bone marrow mesenchymal stem cells$[79]$</td>
<td></td>
</tr>
<tr>
<td>Native chemical ligation</td>
<td>Ethyl 3-mercaptopropionate, Boc-Cys(Trt)-OH</td>
<td>PEG$[82-84]$, Insulinoma cells$[83]$, mesenchymal stem cells$[84]$, induced pluripotent stem cells$[84]$</td>
<td></td>
</tr>
</tbody>
</table>
| Oxime formation                     | Sodium periodate, N-hydroxyphthalimide                                                                    | PEG$[118,119]$, hyaluronic acid$[119]$, alginate$[119]$, mesenchymal stem cells$[118]$}
### Disulfide formation

\[
R_1\text{-SH} + R_2\text{-SH} \xrightarrow{[\text{ox}]} R_1R_2\text{-S-S-S-R}_2
\]

Dithiobis(propanoic or butyric) dihydrazide, N,N′-Bis(acryloyl)cystamine (require a reducing step), thioacetic acid (requires saponification), N-acetyl-L-cysteine or L-cysteine

Hyaluronic acid[120], chitosan[121], PEG[122,123], gellan gum[124], copoly(acrylamide)[125]

Adipose fibroblasts[120]

### Sol-gel

\[
R_1\text{-Si(OEt)}_3 + R_2\text{-Si(OEt)}_3 \rightarrow \begin{array}{c}
\text{Si-O-Si} \\
\text{O} \\
\text{R}_1 \\
\text{O} \\
\text{Si} \\
\text{O} \\
\text{R}_2
\end{array}
\]

3-isocyanatopropyltriethoxysilane, 3-(glycidoxypropyl)triethoxysilane, 3-aminopropyltriethoxysilane

PEG[126,127], gelatin[128,129], chitosan[130–132], collagen[133], alginate[134], hydroxypropylmethylcellulose[135], collagen-inspired undecapeptide[136]

Articular chondrocytes[135,137], cardiomyocytes[138], chondrosarcoma cells[135,139], mesenchymal stem cells[136,140,141]
2.2. Free-radical photopolymerization
Light-activated cross-linking is of particular interest as it offers spatial and temporal control of the hydrogel formation. Indeed, the gelation is triggered by a light beam that can be targeted with high precision, at well-defined times, on the precursor solution. Free-radical photopolymerization is the most common method to prepare hydrogels under physiological conditions.[142,143] Multifunctional macromolecular precursors can undergo chain-growth or step-growth polymerization depending on the chosen functionalizing moieties.

2.2.1. Chain-growth photopolymerization
Vinyl-functionalized macromolecules are subjected to chain-growth polymerization. This process requires a photoinitiator (PI). Upon irradiation at the appropriate wavelength, the photoinitiator absorbs the light energy and dissociates into free-radical reactive species (Fig. 2). These reactive species add to vinyl groups on macromolecular precursors by opening homolytically the π-bond and forming new free radical species. Hence, the reactive center is propagated as covalent bonds are formed between precursors, resulting in the formation of a polymer network within seconds to minutes.

```
PI \xrightarrow{hv} PI' \quad R \quad \equiv \quad R \quad \equiv \quad R
```

*Fig. 2. Chain-growth photopolymerization mechanism. PI: photoinitiator, hv: light source.*

Usually, ultra-violet light is chosen to trigger the polymerization, 365 nm being the most common wavelength.[37] Among the different vinyl groups introduced in precursors (Fig. 3), acrylates are among the most popular. They can be introduced in hydroxyl groups using acryloyl chloride in the presence of a base (e.g. trimethylamine).[38,144] Yang et al. investigated osteogenesis of bone marrow stromal cells in a 3D environment formed by photopolymerization of commercially available PEG-diacrylate (PEGDA) and a PEG acrylate functionalized with peptide ligands promoting cell adhesion.[145] Peyton et al. used the same strategy to study smooth muscle cell behavior in a 3D model system.[146] Methacrylate groups are also suitable for hydrogel photopolymerization. Weber et al. encapsulated murine pancreatic islets in dimethacrylated PEG (PEGDM) hydrogels for insulin-producing cell delivery applications[39] while Russell et al. used it to study the impact of PEGDM rheological properties on oligodendrocyte precursor cell differentiation.[147] PEGDM can be mixed with other synthetic or natural methacrylated polymers. Bryant et al. designed degradable hydrogels by copolymerizing PEG with PLA.[51] The same group also prepared hydrogels from PVA and a natural polymer, chondroitin sulfate. Both polymers were functionalized with methacrylate by reacting their hydroxyl groups with 2-isocyanatoethylmethacrylate and methacrylic anhydride respectively.[52] Poly-D,L-lactic acid/polyethylene glycol/poly-D,L-lactic acid (PDLLA-PEG) was also methacrylated and polymerized to make a biodegradable hydrogel suitable for 3D-culture of MSCs.[148] Methacrylated alginate[40], gelatin[48–50], collagen[45] and hyaluronic acid[41–43] were also described. More recently, a zwitterionic carboxybetaine methacrylate precursor was reported.[149] Diacrylamide groups are less common than (meth)acrylates. However, PEG diacrylamide conjugated to collagen has enabled the preparation of a hydrogel in which the co-culture of both endothelial cells and fibroblasts led to the formation of capillary vessel-like networks.[150] 4-arm PEG-methacrylamide has also been mixed with gelatin-methacrylamide to encapsulate bone marrow mesenchymal stem cells.[151]

It is noteworthy that this type of cross-linking strategy has been applied to 3D bioprinting of cell-embedded hydrogels, as UV-irradiation is now widely implemented in 3D printers. Extrusion-based bioprinting, for example, has been performed with PEG-DMA [152], PEG-DA/acrylated-peptides...
[153], gelatin-MA/PEG-DA [154], Gelatin-MA/hyaluronic acid-MA/4-arm-PEG-acrylate [155], di-acrylated-PCL-PEG-PCL [156] and silk-MA [157]. Furthermore, stereolithography (SLA)/digital light processing (DLP) has been used successfully for gelatin-MA [158,159] and gelatin-MA/glycidal methacrylate-hyaluronic acid [160] bioprinting.

Fig. 3. Chemical structures of vinyl groups used for preparation of hydrogels by chain-growth photopolymerization.

Although this chain-growth photopolymerization cross-linking strategy has been successfully applied to the preparation of numerous cell-laden hydrogels, careful consideration must be taken in choosing the photoinitiating conditions. Investigation of cytotoxicity of several photoinitiators [37,161,162] found cell viability in their presence to be dependent on cell types and cell division rate. Irgacure 2959 (2-hydroxy-1-(4-(2-hydroxyethoxy)phenyl)-2-methylpropan-1-one, Fig. 4) proved to be well-tolerated by a broad range of mammalian cells, and is the most frequently used photoinitiator for cell encapsulation.[38,51,163] Nevertheless, Fedorovitch et al. observed adverse effects of Irgacure 2959-initiated photopolymerization on MSC monolayers.[164] Likewise, Rouillard et al. demonstrated that Irgacure 2959-photogenerated radical species were cytotoxic and led to cell viability below 70%.[40] To overcome these issues, another UV-photoinitiator, lithium phenyl(2,4,6-trimethylbenzoyl)phosphinate (LAP) was developed. Less toxic than Irgacure because of its absorbance in near-UV at 365 nm, it is also highly soluble in water, yielding higher polymerization rates.[165,166]
Another factor to be considered is the use of UV versus visible light. UV photons carry more energy than visible-light photons, which is why UV wavelengths were selected in the first instance for efficient photoactivation. Yet it is well-known that UV exposure can damage cells.[164,167] As a consequence, significant efforts have been made in developing visible light-initiated systems[168], usually using visible blue light. Eosin Y (Fig. 4) and triethanolamine have been used several times as photoinitiator and co-initiator respectively, sometimes with N-vinylpyrrolidone (NVP) to accelerate gelation.[169,170] For instance, this initiator solution allowed the visible-light cross-linking of collagen methacrylamide.[44] Methacrylated glycol chitosan, obtained by reacting commercially available glycol chitosan with glycidyl methacrylate, was photocross-linked at 400-500 nm with the riboflavin photoinitiator.[46,47] Hoshikawa et al. described the photopolymerization of styrene-modified gelatin with camphorquinone photoinitiator.[171]

Cells can be damaged even when visible light is used to initiate the polymerization process. Indeed, during the chain-growth polymerization, molecular oxygen acts as a radical scavenger leading to the formation of reactive oxygen species (ROS). ROS are known to generate oxidative stress in cells.[172] J. J. Roberts and S. J. Bryant showed that chondrocytes exhibited high levels of intracellular ROS immediately after their encapsulation in a PEGDA hydrogel.[173] According to these authors, step-growth polymerization is preferred since this mechanism consumes ROS.

### 2.2.2 Step-growth photopolymerization

Step-growth photopolymerization requires two functional groups, a vinyl and a thiol. Thus, it is often referred to as thiol-ene photopolymerization.[174] The process can be initiated under similar experimental conditions to those of chain-growth photopolymerization (UV or visible light, same photoinitiators). Upon irradiation, the photoinitiator decomposes into free-radical species. A radical attracts a hydrogen atom from a thiol group to produce a thyl radical that reacts with a double bond (Fig. 5). The resulting alkane radical attracts a hydrogen atom from another thiol group, thus forming a thioether bond, and regenerating a thyl radical. The thiol-ene coupling and the thyl radical
generation occur alternatively in a stoichiometric ratio until one of the precursors is entirely consumed. When precursors are multi-functionalized, a 3D network with thioether linkages is formed.

The radical-mediated process can be propagated by ROS which results in low ROS levels in the hydrogel. As a consequence, step-growth polymerization has been found to be cell-friendlier than chain-growth mechanism.[173,175] In addition, PEG hydrogels formed by step-growth mechanisms display superior physical properties than PEG hydrogels obtained by chain-growth mechanism. The resulting network is more homogeneous and has a higher functional group conversion.[175] When (meth)acrylates are chosen as vinyl moieties for thiol-ene photopolymerization, the step-growth mechanism is no longer guaranteed and a mixed-mode photopolymerization can occur. In this case, the free-radical species formed by irradiation of the photoinitiator can react both with the thiol and (meth)acrylate groups leading to a combination of step- and chain-growth polymerization. After studying the formation of thiol-acrylate networks through mixed-mode photopolymerization and investigating the mechanical properties and degradation behavior of the resulting hydrogels,[176] Anseth and co-workers applied this strategy to the encapsulation of human mesenchymal stem cells (hMSCs).[177] The hydrogel was obtained by mixed-mode photopolymerization of PEGDA with cell-adhesion peptides containing a cysteine. Hao et al. used the same strategy with visible-light irradiation.[178] In another study, these authors compared gelation kinetics when using different tetra-functionalized PEG (acrylate, methacrylate, acrylamide and allylether) in a mixed-mode thiol-ene polymerization.[179] More recently, hMSCs were cultured in a hydrogel obtained by visible-light-mediated cross-linking of thiolated heparin and methacrylated hyaluronic acid.[180] The thiol functionalized heparin was prepared by converting carboxyl groups of heparin into thiol groups while methacrylate groups were introduced into hyaluronic acid by reaction with methacrylic anhydride. Alternatively, commercial thiolated hyaluronic acid has been cross-linked with hyper-branched PEG-diacrylate to encapsulate MSCs.[181] The primary alcohol functions of hyaluronic acid were reacted with pentenoic anhydride to yield pentenoate functionalized HA, which could be cross-linked with dithiothreitol after light irradiation at 312 nm of Irgacure 2959 allowing cell encapsulation.[182] HA functionalized with allyl glycidyl ether, was mixed with dithiothreitol and chondrocytes, to be 3D bioprinted by extrusion (followed by UV-irradiation) or DLP.[183]

To ensure a step-growth mechanism, a thiol-norbornene combination can be chosen for thiol-ene photopolymerization.[184] This system was developed by Fairbanks et al.[53] They first described the preparation of a proteolytically degradable hydrogel by step-growth photopolymerization of a norbornene-functionalized 4-arm PEG (Fig. 6) and a chymotrypsin-degradable peptide containing two cysteines. The norbornene moieties were introduced on PEG by coupling norbornene acid activated as a symmetrical anhydride. The thiol groups derived from the side-chains of the cysteines. hMSCs were successfully encapsulated in this hydrogel using Irgacure 2959 (Fig. 4) as the photoinitiator with irradiation at 352 nm. Aziz et al. also used this type of hydrogel functionalized with a cysteine-bearing-RGD sequence, and cross-linked through dithiol-MMP-sensitive peptide sequences, for the encapsulation of hMSCs.[185] Lin et al. reported a similar system with irradiation at 365 nm.[175] Ooi et al. also used 365nm irradiation to cross-link alginate-norbornene with

Fig. 5. Step-growth photopolymerization mechanism. PI: photoinitiator, hv: light source.
thiolated-PEG and thiolated-RGD, after extrusion-bioprinting.[165] The thiol-norbornene photopolymerization was then adapted for initiation with visible light.[186,187,55,56] In this system, homopolymerization between norbornenes does not happen which guarantees a step-growth mechanism. In numerous examples, bioactive peptides have been introduced into one-functionalized polymer networks taking advantage of the cysteine thiol group. The mirror reaction is also possible as demonstrated by reacting allyloxycarbonyl (Alloc) -protected amino-acids with thiol-functionalized star-PEG.[188]

![Fig. 6. 4-arm PEG norbornene.](image)

Together, these examples all demonstrate the utmost importance of free-radical photopolymerization for the preparation of cell-laden biomimetic hydrogels. The main advantage of this cross-linking strategy is the spatial and temporal control over gelation kinetics. The main limitation of this method is the adverse effects on encapsulated cells. Whatever the polymerization mechanism (chain- or step-growth) and the wavelength used for irradiation, the radical species involved may interact with encapsulated cells and be detrimental to their survival and differentiation. Also of interest is the covalent functionalization of hydrogels with bioactive molecules. It is theoretically possible to mix hydrogel precursors with either vinyl-modified or thiol-containing molecules to covalently attach these molecules to the network during its formation by photopolymerization. However, the immobilization efficiency of pendent mono-functionalized bioactive molecules on the cross-linked network is often rather low and the unreacted free bioactive molecules can lead to undesired biological responses. To overcome this issue, two separate chemistries have to be used: the first one to conjugate the bioactive molecule to multifunctional macromolecular precursor and the second one to cross-link these precursors.

### 2.3. Click chemistry

Click chemistry was first defined by Sharpless and co-workers in 2001.[189] Although this term immediately brings to mind the azide alkyne cycloaddition, it is not restricted to this reaction. Indeed, it refers to a whole class of modular reactions that rapidly proceed to completion with high selectivity for a single reaction product. These reactions are characterized by a high thermodynamic driving force. Insensitive to oxygen and water, they occur one-pot to provide the desired product in high yields. Only inoffensive by-products are formed. These criteria allow click reactions to occur in the presence of living systems without interfering with biological processes (bio-orthogonality) and thus made click reactions ideal candidates for biomimetic hydrogel preparation.[190–193] Amide-forming reactions, while extensively used to synthesize various bioconjugates through carboxylic acid activation (such as N-hydroxysuccinimidy esters (NHS)), will not be discussed here, as they are not bioorthogonal. Indeed they should be avoided in the preparation of cell-encapsulating hydrogels, given that cells and the cell culture medium contain reactive amines. This theoretical requirement was not always taken into consideration as robust cells lines (HeLa and 3T3 fibroblasts) were encapsulated
in polyamidoamine dendrimer cross-linked by PEG-NHS chemistry with rather good cell viability (80%) after two days.[194]

2.3.1. Michael-type addition
Michael addition is the 1,4- addition of a nucleophile to an α,β-unsaturated system (Fig. 7). This conjugate addition was discovered by Arthur Michael in 1887.[195] It involves a Michael donor, the nucleophile, and a Michael acceptor, the α,β-unsaturated system. Enolates and amines can be used as Michael donors but thiols are preferred due to their higher nucleophilicity and selectivity under physiological conditions.[196,197] Michael acceptors are electron-deficient ene such as (meth)acrylates, acrylamides, vinyl sulfones and maleimides (Fig. 8). Although the Michael addition of a thiol precursor to an activated double bond yields a thioether, the reaction mechanism is completely different from the thiol-ene photopolymerization described above, which involves radical species.

\[
\text{R-SH} + \text{ EWG} \rightarrow \text{ R-S-EWG}
\]

Michael donor Michael acceptor

Fig. 7. Michael-type addition involving a thiol as the Michael donor. EWG: Electron-withdrawing group.

\[
\begin{align*}
\text{methacrylates} & \quad \text{acylates (}X=\text{O}) \\
\text{acrylamides (}X=\text{NH}) & \quad \text{vinyl sulfone} \\
\text{maleimide} &
\end{align*}
\]

低 REACTIVITY 高

Fig. 8. Michael acceptors and their reactivity towards thiols.[197]

Hubbell and colleagues pioneered the use of Michael-type additions to prepare cell-encapsulating hydrogels. They reported hydrogels resulting from the cross-linking of branched PEG multi-vinyl sulfone with a MMP-sensitive peptide containing two cysteines (e.g., Ac-GCRD-GPQG↓IWGQ-DRCG).[198,199] Vinyl sulfone groups were introduced by reacting divinylsulfone with PEG treated with sodium hydride. Acrylated polymers were also used as Michael acceptors to prepare hydrogels.[200] In particular, chondrocytes and embryonic stem cells were encapsulated in hydrogel formed by cross-linking thiol-functionalized dextran with a tetra-acrylated 4-arm PEG.[201] As another example, Zheng Shu et al. investigated the reaction of thiol-functionalized hyaluronic with PEG diacrylate, dimethacrylate, diacrylamide and dimethacrylamide in order to encapsulate fibroblasts.[202] More recently, Speidel et al. described the encapsulation of cardiac stem cells in a hydrogel made of 4-arm PEG cross-linked by heparin-binding peptides via Michael addition with cysteines.[203] To increase degradability, Stevens et al. introduced acrylate function in collagen through NHS-PEG-acrylate cross-linked in the presence of hMSCs, with thiolated MMP-degradable peptides.[204,205]

Triethanolamine and HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) have often been used as catalysts to facilitate the Michael addition reaction.[206] At high concentrations, these compounds might be toxic to cells. To overcome this issue, Garcia et al. investigated the reactivity of PEG maleimide, PEG vinyl-sulfone and PEG-acrylate as Michael acceptors. They demonstrated
that PEG maleimide afforded the fastest reaction kinetics and required less triethanolamine to form a gel, resulting in a higher encapsulated cell viability.[58] They illustrated these results through the encapsulation of pancreatic islets in a hydrogel obtained by Michael addition of a cross-linking degradable peptide containing 2 cysteines on a 4-arm PEG tetra-maleimide (Fig. 9). In this example, the Michael addition chemistry was also used to functionalize the hydrogel with a cell-adhesion peptide and VEGF.[57] Recently, Lim et al. prepared an hydrogel from a cysteine-modified protein (modified gamma-prefoldin (SpyTag–γPFD–Cys)) cross-linked with a 4-arm PEG maleimide to encapsulate human embryonic stem cells.[207] The same strategy was used elsewhere, replacing the protein by thiolated hyaluronic acid and adding cysteine-containing adhesion peptides (RGD, IKVAV and YIGSR sequences). 3D culture of H9 human embryonic stem cells was performed in these gels.[208]

![Diagram of Michael addition and peptide conjugation](image)

**Fig. 9.** Example of a functional hydrogel prepared by Michael addition.[57]

Baldwin and Kiick showed that the succinimide thioether linkage resulting from the Michael addition of thiols to maleimides could undergo retro and exchange reactions at physiological pH and temperature.[209] It is possible to stabilize the thioether linkage by opening the succinimide ring. Alternatively, the authors proposed taking advantage of these properties to control the release of a drug and to tune the degradability of the hydrogel.[59]

Although the thiol-Michael addition is attractive for the design of biomimetic hydrogels, a limitation of this strategy can arise from the use of thiol-functionalized molecules for *in vivo* gelation. Indeed, thiols are naturally occurring chemical groups. Thus, their administration *in vivo* can lead to undesired cross-reactivity, oxidation or metabolism.
2.3.2. Azide-alkyne cycloaddition

Cycloaddition of monosubstituted alkynes and azides gives 1,2,3-triazoles. While the thermal Huisgen 1,3-dipolar cycloaddition often produces a mixture of two regioisomers, the copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) gives selectively 1,4-disubstituted regioisomers (Fig. 10).[210]

\[
R-N_3 + ==-R' \xrightarrow{\Delta} \text{Huisgen 1,3-dipolar cycloaddition}
\]
\[
R-N_3 + ==-R' \xrightarrow{\text{Cu(I) cat.}} \text{Copper(I)-catalyzed alkyne azide cycloaddition}
\]

Fig. 10. Alkyne-azide cycloadditions yielding 1,2,3-triazoles.

This reaction can proceed under physiological conditions and is bio-orthogonal since it involves two mutual reactive groups, azides and alkynes, which are not found naturally in living cells. Cu(I) species can be used directly to catalyze the reaction. Usually, however, the Cu(I) active catalyst is generated \textit{in situ} from Cu(II) salts using a reducing agent such as sodium ascorbate. Alternatively, Adzima et al. demonstrated that Cu(II) could be photochemically reduced to Cu(I), affording spatial and temporal control over CuAAC-based gelations.[211] In contrast to the concerted Huisgen cycloaddition mechanism, CuAAC proceeds through a stepwise mechanism (Fig. 11).[212] It starts with the formation of a copper acetylide. Then, the azide binds to the copper acetylide and a six-membered copper(III) metallacyle forms. Ring contraction to a triazolyl-copper derivative and protonolysis yield the 1,4-disubstituted 1,2,3-triazole derivative.

Fig. 11. Proposed mechanism for the Copper(I)-catalyzed alkyne-azole cycloaddition.[212] L: ligand, H$_2$O for example.
Ossipov and Hilborn were the first to apply CuAAC to the preparation of hydrogels using alkyne and azide-functionalized PVA and PEG.[213] Alkyne and azide groups were introduced on polymers by reaction with propargylamine derivatives and either 2-azidoethylamine or sodium azide respectively. Several hydrogels have been prepared with the CuAAC strategy,[214–218] but only Crescenzi et al. reported the encapsulation of cells in a CuAAC cross-linked hyaluronan hydrogel.[219] Indeed, the major limitation of CuAAC strategy is the cytotoxicity of copper ions and their ability to generate ROS in vivo.[220]

To avoid the use of toxic copper ions, an alternative, metal-free approach was developed in which ring strain was used to promote the azide-alkyne cycloaddition.[221] The reaction between a strained cyclooctyne and an azide, called strain-promoted alkyne-azide cycloaddition (SPAAC), proceeds under physiological conditions without additional reagent (Fig. 12).

**Fig. 12.** Strain-promoted azide alkyne cycloaddition (SPAAC) between an azide and a cyclooctyne.

SPAAC has been successfully applied to the preparation of injectable hydrogels[60] and cell-encapsulating hydrogels. DeForest and Anseth have worked extensively on SPAAC cross-linking.[61–66] In their system, gelation was achieved by a SPAAC reaction between a cyclooctyne-functionalyzed 4-arm PEG and a bis(azide)-functionalyzed peptide,[63,64,66] or alternatively between a 4-arm PEG azide and a cyclooctyne-functionalyzed peptide.[61,62] For most of their studies,[61–64] DeForest and co-workers chose a gem-difluoro cyclooctyne (DIFO, Fig. 13) since the presence of strong electron withdrawing fluorines enhanced the SPAAC kinetics and afforded a faster gelation.[222] Dibenzylcyclooctyne-functionalyzed PEG were also reported for hydrogel preparation allowing cell encapsulation.[67,68,65] Zhan et al. used a star-PEG-bicyclo[6.1.0]nonyne (BCN, Fig. 13) with a 4-arm-azide-star-PEG-containing MMP-sensitive sequences to create an enzymatically-degradable hydrogel by a copper-free SPAAC, allowing encapsulation of HeLa cells.[69]

**Fig. 13.** Structure of activated yne groups used for metal-free azide-alkyne cycloaddition.

The main limitation of the SPAAC cross-linking strategy comes from the synthesis of cyclooctyne derivatives that involves over 10 steps with a low overall yield. Recently, Truong et al. overcame this issue by taking advantage of the reaction of azides with propiolic esters (Fig. 13).[223] In propiolic esters, the triple bond is activated by the neighboring electron-withdrawing ester group. The electron-deficiency of the alkyne allowed the cycloaddition to proceed without catalyst. Truong et al. demonstrated that a cell-laden hydrogel could be obtained from 3-arm PEG propiolate and azide-functionalyzed chitosan.[224] Proceeding under physiological conditions between two functions that are not found in the biomolecules of living organisms without any additional reagent, the cycloaddition between azides and propiolic esters appears to be a promising tool for biomimetic hydrogel preparation.

### 2.3.3. Diels-Alder cycloaddition

The Diels-Alder reaction (DA) refers to a [4+2] cycloaddition of a conjugated diene to a dienophile (Fig. 14). Ideally, it occurs between an electron-rich diene and an electron-deficient double bond. It was first reported in 1928 by Otto Diels and Kirk Alder.[225] The reaction is spontaneous under...
physiological conditions. Neither catalyst nor additional reagent is needed, and no by-product is formed. It proceeds through a concerted mechanism and the reaction rate is advantageously enhanced in water compared to organic solvents.[226,227]

**Fig. 14.** Diels-Alder reaction. EDG: Electron-donating group, EWG: Electron-withdrawing group.

Furan and maleimide are the most commonly used diene and dienophile for the preparation of Diels-Alder cross-linked hydrogels (Fig. 15).[71,72,76,228,73,74,229] Yu et al. functionalized hyaluronic acid (HA) with furylamine, after activation of HA carboxyl groups with (4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholiniumchloride (DMTMM). The resulting HA-furan and the commercially available bis(maleimide)-PEG underwent Diels-Alder reaction in DPBS. Murine chondrocytic cells were successfully encapsulated during the process.[70]

**Fig. 15.** Diels-Alder reaction between furane and maleimide moieties.

Furthermore, it has been shown that the Diels-Alder adducts were reversible, and that the equilibrium could be shifted towards the reverse Diels-Alder reaction under physiological conditions.[230] This reversibility was advantageously used to modulate the degradation of hydrogels,[75] to promote the controlled release of a drug[231,232] and to prepare self-healing hydrogels.[77]

The click Diels-Alder reaction with its reversibility seems attractive for the preparation of engineered biocompatible hydrogels. Yet, very few authors reported direct encapsulation of cells in the gels using the Diels-Alder strategy. This may be attributed to rather long gelation time (> 50 min)[70]. Another limitation could arise from the reactivity of maleimide towards thiol-containing biomolecules in vivo, undergoing Michael-type 1,4-addition yielding thioether ligation products.

Notably, a second type of Diels-Alder cycloaddition was investigated as a coupling tool: the inverse electron demand Diels-Alder reaction.[233,234] This reaction involves an electron deficient diene and an electron rich dienophile to yield a formal [4+2] Diels-Alder adduct (Fig. 16). 1,2,4,5-Tetrazines are the most popular dienes for this reaction. In the presence of strained dienophiles such as norbornene or trans-cylooctene, they undergo an inverse electron demand hetero-Diels-Alder reaction, instantly followed by a retro Diels-Alder reaction releasing nitrogen (Fig. 17).

**Fig. 16.** Inverse electron demand Diels-Alder reaction. EDG: Electron-donating group, EWG: Electron-withdrawing group.

**Fig. 17.** Inverse electron demand hetero-Diels-Alder reaction. EDG: Electron-donating group, EWG: Electron-withdrawing group.
Fig. 17. Tetrazine-norbornene inverse electron demand hetero Diels-Alder-retro-Diels-Alder cascade.

Anseth and fellow researchers applied the inverse electron demand Diels-Alder reaction to the preparation of cell-encapsulating hydrogels. The hydrogel formed within minutes from a multi-arm PEG tetrazine and a cell degradable dinorbornene cross-linker peptide (norb-KGPQGIWGQKK-norb). When functionalized with a norbornene-bearing cell adhesion peptide, these hydrogels allowed the encapsulation of hMSC with high viability.[78] In another example, Jia and Fox encapsulated prostate cancer LNCaP cells in hydrogel microspheres by reacting tetrazine-modified hyaluronic acid with a bis-trans-cyclooctene cross-linker.[80] They also decorate this hydrogel with TCO-functionnalized RGD and MMP-degradable peptides to create tunable hydrogel for hMSCs culture.[81] Zhang et al. also used tetrazine-modified HA, but cross-linked it with 4-arm PEG-trans-cyclooctene in the presence of bone marrow mesenchymal stem cells.[79]

2.3.4. Native chemical ligation

Native chemical ligation (NCL) was initially developed for polypeptide and protein syntheses.[235] This method allows the coupling of two unprotected peptide sequences through the formation of a peptide bond. Generally, the reaction occurs between a C-terminal thioester peptide and an N-terminal cysteine residue from the other peptide. The first step is a reversible, chemoselective and regioselective transthioesterification (Fig. 18). The thiol side-chain of the cysteine residue attacks the thioester to form a thioester intermediate. Then, an intramolecular S→N acyl shift results in the formation of a ‘native’ amide bond.

![Fig. 18. Native chemical ligation.](image)

Messersmith and co-workers were the first to describe hydrogels cross-linked by NCL.[82] They prepared thioester-terminated PEG by coupling ethyl 3-mercaptopropionate succinic acid onto PEG amine. Cysteine-terminated PEGs were obtained by coupling either Boc-Cys(Trt)-OH or the dipeptide Boc-Cys(Trt)-AA-OH onto PEG amine followed by Boc removal in acidic conditions. Later on, they reported encapsulation of pancreatic islets and stem cells in these PEG-based hydrogels.[83,84] Notably, NCL ligation has also been used to chemically cross-link physical hydrogels.[236]

The oxo-ester mediated chemical ligation (OMNCL) was proposed as a variant of the NCL reaction, in which thioesters were replaced by activated oxo-esters such as para-nitrophenyl ester.[237] Messersmith and colleagues applied this reaction to the cross-linking of hydrogels.[238] They successfully encapsulated cells in an OMNCL cross-linked hydrogel. The hydrogel network resulted from the amide bond formation between 8-arm PEG functionalized with N-hydroxysuccinimide oxoesters and cysteines via an OMNCL reaction. However, due to the reactivity of the para-nitrophenyl ester, side products generated by direct nucleophilic addition of primary amino groups (lysine side chains, protein N terminus) were highly probable.

NCL is still under investigation as a cross-linking method. The hydrolytic instability of thioesters may be a limitation for its applications. In addition, the use of free sulfhydryl cysteine residues may require the presence of reducing agents to prevent formation of disulfide bonds. It may also lead to cross-reactions in vivo with cysteine-containing proteins, which are commonly present in the cell membrane. Last but not least, special attention has to be paid to the thiol leaving group that could generate adverse biological effects.
2.3.5. Imine, hydrazone and oxime formation

Imines, hydrazones, acylhydrazones and oximes are formed through the reversible reaction of a carbonyl group (an aldehyde or a ketone) with an amino group (a primary amine, a hydrazine, a hydrazide or a hydroxylamine respectively) (Fig. 19). These reactions expand the toolbox of cross-linking strategies. Aldehydes are usually chosen as carbonyl groups because of their high reactivity towards nucleophiles compared to ketones.

\[
\begin{align*}
\text{A} & \quad R^1CH=O + H_2N^+R^- \rightleftharpoons R^1CNH^+R^- + H_2O \\
\text{amine} & \quad \text{imine} \\
\text{B} & \quad R^1CH=O + H_2N^+R^- \rightleftharpoons R^1CNH^+R^- + H_2O \\
\text{amine} & \quad \text{imine} \\
\text{hydrazine} & \quad \text{hydrazone} \\
\text{C} & \quad R^1CH=O + H_2N^+R^- \rightleftharpoons R^1CNH^+R^- + H_2O \\
\text{amine} & \quad \text{imine} \\
\text{hydrazine} & \quad \text{hydrazone} \\
\text{D} & \quad R^1CH=O + H_2N^+O^-R^- \rightleftharpoons R^1CNH^+O^-R^- + H_2O \\
\text{amine} & \quad \text{imine} \\
\text{hydroxylamine} & \quad \text{oxime} \\
\end{align*}
\]

Fig. 19. Reaction of an aldehyde with a primary amine, a hydrazine, a hydrazide and a O-substituted hydroxylamine to form an imine, a hydrazine, an acylhydrazone and an oxime respectively.

Hydrogel preparation through imine formation, also known as Schiff’s base formation, is limited by the low hydrolytic stability of imine bonds. Nevertheless, it has been investigated by several groups.[85,87,93,96,90,91,97,86,89] In particular, Tan et al. developed an imine cross-linked hydrogel for cartilage tissue engineering. The hydrogel was obtained from modified chitosan and aldehyde-functionalized HA.[94] Weng et al. described in situ gelable hydrogels by imine formation between aldehyde groups from oxidized dextran and amino groups from N-carboxyethyl chitosan.[88] Most of the time, imine cross-linked hydrogels involved aldehyde groups from an oxidized polysaccharide since such polysaccharides were easy to access. But synthetic aldehyde derivatives could also be used. For instance, Yang et al. reported an injectable cell therapy carrier hydrogel by cross-linking glycol chitosan with a bis(formyl)PEG.[92] Another injectable hydrogel obtained from aldehyde-modified poly(L-glutamic acid) and modified chitosan was described by Yin’s team.[95,239]

Unfortunately, this strategy is not properly bio-orthogonal. Indeed, the nucleophile used is a primary amine which, as already stated in this manuscript, is commonly found in proteins (lysine side chains and N terminus). Thus, the use of more potent nucleophiles at physiological pH (hydrazines, hydrazides, O-substituted hydroxylamines) seems more attractive to increase the selectivity of the reaction, and minimize unwanted biological material-reagent side reactions.

(Acryl)hydrazone bonds exhibit higher hydrolytic stability than imines and thus afford a hydrogel degradation rate more compatible with drug delivery and tissue engineering applications.[240] Hydrazone and acylhydrazone cross-linking has been widely used for injectable hydrogel preparation.[107,109,113,241,114–116,99] As far as 3D culture systems are concerned, McKinnon et al. introduced a hydrogel to 3D culture mouse myoblasts made from hydrazine and aldehyde-
modified multi-arm PEGs, in combination with an aldehyde-functionalized cell adhesion peptide (benzaldehyde-KGRGDS).[100] Then, they showed how this hydrogel could be used to study the biophysical forces involved in neurite extension.[98] Following the work of Ossipov et al.,[103] Martens and colleagues reported cell encapsulation in acylhydrazone cross-linked PVA or PVA-heparin hydrogels.[102,242] Gurski et al. developed a 3D matrix for in vitro anti-cancer drug screening and the study of cancer cell motility. The system consisted of an acylhydrazone cross-linked HA hydrogel encapsulating cancer cells.[105,106] Wang et al. demonstrated the feasibility of 3D-bioprinting fibroblast encapsulated inside this hydrogel.[243] Many more examples of (acyl)hydrazone cross-linked hydrogels exist.[108,110,111,117,101,244–246] As a final illustration, we can mention the hydrogels developed by Dahlmann et al. for myocardial tissue engineering. They produced spontaneously contracting bio-artificial cardiac tissues by encapsulating neonatal rat heart cells in acylhydrazone cross-linked alginate-HA hydrogels containing human type I collagen.[112]

Roots are close relatives to imines and hydrazones but they exhibit a higher hydrolytic stability.[247] They have received little attention as cross-linkages. Grover et al. worked on oxime cross-linked hydrogels.[118,119] They cross-linked an aminooxy-8arm-PEG with glutaraldehyde in the presence of a ketone-containing cell adhesion peptide (5-aminolevulinic acid-GRGDSPG). Although glutaraldehyde is known to be cytotoxic,[248,249] they showed that MSC could be encapsulated in this hydrogel with high viability.[118]

Due to their reversibility, imine, hydrazine, acylhydrazone and, to a lesser extent, oxime bonds are dynamic cross-links. They can provide self-healing properties to materials and enable a controlled degradation.[250] Aldehyde groups are preferred to ketones for their higher reactivity. However, because of this high reactivity, aldehydes can react in vivo with amines from biomolecules and lead to toxicity.

### 2.3.6. Disulfide formation

Disulfide bonds play a key role in protein folding and stability.[251,252] They result from the oxidation of two thiol groups (Fig. 20). Mild oxidizers, such as O2, are sufficient to drive the oxidative coupling. Thus, cross-linking of thiol-containing polymers can be achieved under physiological conditions with atmospheric oxygen. Prestwich and co-workers functionalized hyaluronic acid with dithiobis(propanoic dihydrazide) and dithiobis(butyric dihydrazide).[120,253] The initial disulfide bonds were reduced with dithiothreitol to give thiolated HA that could turn into a hydrogel upon oxidation of the thiol with air. They demonstrated that encapsulated L929 fibroblasts could proliferate in this disulfide cross-linked HA hydrogel.[120] Liu et al. used this hydrogel with addition of thiolated-icariin, a flavonoid facilitating the chondrogenesis of mesenchymal stem cells (MSCs).[254] Chitosan,[121] PEG[122] and gellan[124] were also modified by thiol moieties and cross-linked into hydrogels. Thiol groups were often introduced via the coupling of a cysteine residue. However, Swindle-Reilly et al. prepared hydrogels from thiolated poly(acrylamide) obtained by copolymerization of acrylamide and bisacryloylcystamine.[125]

$$\text{R-SH} + \text{R'-SH} \xrightarrow{[\text{ox}]} \text{R-S-S-R'}$$  

**Fig. 20.** Disulfide bond formation and reduction.

Cross-linking of hydrogels through oxidation of free thiols often requires long gelation times. Alternatively, polymers can be cross-linked via thiol-disulfide exchange reaction (Fig. 21).[255] This reaction is a nucleophilic substitution between a thiol and an activated disulfide that leads to the formation of a new disulfide and a thiol. Unlike the disulfide bond formation from sulfhydryl derivatives, this strategy ensures the formation of intermolecular linkages, avoiding the risk of intermolecular cyclized side products. Choh et al. used pyridyl-disulfide-modified HA and PEG
dithiol to encapsulate several cell types in a disulfide cross-linked hydrogel.[256] In this case, cellular effects of thiols released during thiol-disulfide exchange reactions have to be carefully studied.

![Thiol-disulfide exchange reaction](image)

**Fig. 21.** Example of thiol-disulfide exchange reaction used by Choh and co-workers to prepare a hydrogel.[256]

Lee *et al.* showed that compression or tension applied to PEG hydrogels with disulfide linkages induced disulfide bond rupture. They took advantage of the compression-induced generation of free sulfhydryl groups to functionalize these hydrogels with a maleimide-containing fluorophore through a Michael-type addition.[123] Nonetheless, disulfide bond rupture in response to applied force could be limiting for applications of disulfide cross-linked hydrogels as ECM substitutes for tissues subjected to high physical forces. In addition, disulfide cross-linked hydrogels are rapidly reduced by glutathione [H-Glu(CysGlyOH)-OH], a naturally occurring anti-oxidant tripeptide synthesized in human cells. Therefore, they are more appropriate for short-term applications. As already mentioned for Michael-type additions and native chemical ligation, thiols can lead to cross-reactivity with proteins and peptides in vivo.

Overall, the application of click reactions to the cross-linking of hydrogels has led to ground-breaking development in the field of biomaterials. However, the design of cell-encapsulating hydrogels remains a challenge. Some other bio-orthogonal ligation reactions are only starting to be explored by a few groups. For instance, Stabler *et al.* worked on Staudinger ligation and showed that this reaction could be advantageously used to encapsulate pancreatic islets in alginate-PEG hydrogels.[257,258] Tamate and co-workers cross-linked a hydrogel through photoinduced dimerization of coumarin moieties.[259] Zong *et al.* have focused their efforts on tetrazole-alkene photo-click chemistry but have not yet proved that this method allows cell encapsulation.[260,261] Reversible covalent bonds such as phenylboronate–salicylhydroxamate cross-links and boronic ester bonds have also been sporadically investigated to design hydrogels with dynamic properties.[262]

### 2.4. Enzyme-mediated cross-linking

It is well known that enzymes catalyze specific reactions with high selectivity and efficiency. Their potential has been used in chemical synthesis as a convenient alternative to traditional organic chemistry.[263,264] As enzymes work under physiological conditions, they are suitable for biocompatible cross-linking strategies.[265]
Enzymes can be used in two ways for hydrogel formation: either as a means to generate initiators in situ for free-radical polymerization of hydrogel precursors, or to catalyze specific covalent bond formation between two mutually reactive moieties displayed by hydrogel precursors.

2.4.1. Enzyme-triggered free-radical polymerization

The oxidation of glucose by the glucose oxidase enzyme (GOx) produces hydrogen peroxide. In the presence of Fe\textsuperscript{2+}, hydrogen peroxide generates hydroxyl radicals that can initiate polymerization through the mechanism described above in section 2.2.1. (Fig. 22, pathway A). Bowman and Anseth used GOx to cross-link PEGDA.[266–268] This system was applied to the coating of cell-encapsulating hydrogels but was never described as a cross-linking method to prepare cell-laden hydrogels.[267] Recently, Wei et al. combined two enzyme-mediated redox reactions in a single system (Fig. 22, pathway B). The hydrogen peroxide released by GOx oxidation of glucose was used by horseradish peroxidase (HRP) to oxidize acetyl acetone, a diketone mediator, as a ternary initiating system to trigger the radical-mediated polymerization of PEG methacrylate (Fig. 22).[269] In these cases, enzymes are not directly involved in the cross-linking process, they are used as initiators for chain-growth polymerization. In that sense, enzyme-triggered free-radical polymerization cannot be properly considered as an enzyme-mediated cross-linking method.

Fig. 22. Enzyme-mediated redox reactions triggering free-radical polymerization

2.4.2. Enzyme-mediated ligation

The potential of enzymes can more usefully be used to catalyze the reaction between two reactive groups leading to the formation of a cross-link.

Horseradish peroxidase (HRP), reported above for triggering free-radical polymerization, can also catalyze the oxidative coupling of phenol moieties in the presence of hydrogen peroxide. A cross-link is formed between two carbon-centered radicals at the ortho-position to the phenolic hydroxyl groups, or between an ortho-carbon radical and a phenolic hydroxyl group (Fig. 23). Wang et al. functionalized gelatin with 3-(4-hydroxyphenyl)propionic acid (HPA) (Fig. 24) and reported the encapsulation of hMSCs in a hydrogel formed by the HRP-catalyzed cross-linking of HPA groups.[270] Likewise, Menzies et al. prepared cell-laden hydrogels from branched-PEG-HPA.[271] Tyramine and tyrosine derivatives can also undergo HRP-mediated cross-linking.[272–274] For instance, Chen and co-workers encapsulated chondrocytes in a HRP cross-linked hydrogel obtained from carboxymethyl pullulan-tyramine and chondroitin sulfate-tyramine.[275] With the same strategy, human MSCs were encapsulated in a HRP cross-linked hyaluronan-tyramine hydrogel.[276] More recently, Nguyen et al. used HRP/H\textsubscript{2}O\textsubscript{2} to cross-link 8-arm PEG-norbornene with bis-cysteine-bearing peptides in presence of murine NIH/3T3 fibroblasts.[277]
Tyrosinase is another oxidative enzyme able to cross-link phenol and catechol-functionalized molecules in the absence of hydrogen peroxide (Fig. 25). This is of interest given the adverse cellular effects of H$_2$O$_2$. However, few studies have investigated tyrosinase-mediated cross-linking for biomedical applications.[278–280] Das et al. reported the encapsulation of mesenchymal progenitor cells in a silk fibroin-gelatin hydrogel. In this study, mushroom tyrosinase was used to create cross-links between tyrosine residues of silk and gelatin.[281] Tyramine and DOPA derivatives were also used to enable tyrosinase-mediated cross-linking.[278,280] Notably, the oxidative coupling of DOPA-bearing polymers can also be achieved with a chemical oxidant such as sodium periodate.[278,282–284]
Transglutaminase (TG) cross-links peptides and proteins by catalyzing an acyl-transfer between the side-chain of a glutamine residue and the side-chain of lysine residue in the presence of its cofactor, Ca$^{2+}$ ion (Fig. 26). This reaction is highly selective for the two substrates (i.e. the lysine containing sequence acting as acyl acceptor and the glutamine-containing peptide acting as acyl donor). Thus, macromolecules that are functionalized with two rationally designed TG substrates can be cross-linked by TG. This strategy was used by Hu and Messersmith, who conjugated TG peptide substrates with PEG chains and demonstrated hydrogel formation under physiological conditions.[285] Prior to this study, Sperinde and Griffith had already obtained TG cross-linked hydrogels from a glutaminamide-functionalized PEG and a poly(LysPhe) peptide.[286] McHale et al. reported chondrocyte encapsulation in an elastin-like polypeptide hydrogel with TG–mediated gelation.[287] A thermally responsive gelation system was developed by Sanborn and co-workers based on the controlled release of the enzyme cofactor. Calcium-loaded liposomes allowed TG activation only when the system temperature was raised to 37°C, releasing the calcium ions during the phase transition of the liposome.[288] Finally, Ehrbar et al. engineered cell-laden hydrogels whose network was formed by combining different molecules functionalized with TG peptide substrates, in particular multi-arm PEGs, cell adhesion peptide and MMP-sensitive peptide.[289–291] Interestingly, the latter lent the resulting network tunable enzymatic degradation properties. With a different goal, Zhou et al. used TG to cross-link methacryloylated gelatin, to tune the rheological properties of a bioink encapsulating C2C12 Myoblasts before its extrusion and UV-cross-linking.[292]
Thrombin is an enzyme involved in the clotting reaction of blood. It has the ability to cleave the N-terminus part of its alpha and beta chains of fibrinogen, a hexameric glycoprotein, forming fibrinopeptides side products. This cleavage leads to the self-assembly of its D and E domains resulting in polymerization, turning it into fibrin fibers (Fig. 27). Cox et al. used this highly specific reaction to encapsulate dermal fibroblast[293], while Dare et al. used it to prepare hydrogels allowing chondrogenic differentiation.[294]

Finally, the phosphopantetheinyl transferase (PPTase) was also successfully used for tissue engineering applications. This enzyme catalyzes the covalent attachment of the 4-phosphopantetheine moiety of coenzyme A (CoA) to a specific serine residue on the apo-acyl carrier protein (ACP) (Fig. 28). Hydrogels were formed from 3-arm PEG multi-functionalized with CoA and an engineered ACP dimer. The PPTase-mediated cross-linking was conducted in the presence of cells, demonstrating its biocompatibility.[295]
Examples discussed in this section show that enzymes are well-suited for the cross-linking of hydrogels. Nevertheless, special attention must be paid to enzymes that work in an oxidative medium either because they require H\textsubscript{2}O\textsubscript{2} or because they generate it.[296] In addition, the enzymes used for cross-linking are either not physiologically present in cell culture media or they are present in too low quantities to avoid their additional introduction during hydrogel formation. It implies that they could lead to immune responses and cross-reactions \textit{in vivo}. Finally, diffusion of enzymes in the medium during the gelation process is limited, which often results in heterogeneous hydrogels with poor mechanical properties.

### 2.5. Genipin-mediated cross-linking

Genipin is an aglycone moiety derived from an iridoid glycoside extracted from \textit{Gardenia jasminoides}' fruit. This natural molecule has the ability to spontaneously cross-link two amino groups of proteins or polysaccharides. When used at 0.5 mM concentration and lower, it is considered biocompatible.[297] Interestingly, the reaction with amines occurs in physiological conditions without the need of any other reagent or catalyst. The genepin core is then included into the network, bridging two (bio)polymer chains (Fig. 29) and yielding a blue coloration due to the conjugate unsaturated bonds of the resulting bicycle. It is noteworthy that the reaction can be monitored by absorbance or fluorescence emission.[298] The mechanism proposed involves first the attack of the double bond of the dihydro-pyranol by an amine to give - after ring opening and water elimination - an aldehyde and a secondary amine. Finally, the lone electron pair of the amine can react with the aldehyde leading to water elimination and dihydro-pyridine formation. At the same time, another amine of the biomolecule can attack the ester, resulting in an amide bond and methanol release.[299,300] Special attention must be paid to the number of equivalents used in the cross-linking reaction, avoiding increasing it by much, as the genipin may also dimerize.[299]
Cross-linking with genipin in the presence of cells has been done with chitosan,[301–303] fibrin,[304] and collagen[298]. All of these hydrogels were prepared for tissue engineering applications such as spinal cord injury repair[298] or cartilage regeneration[304].

2.6. Sol-gel chemistry
Pioneered in the 19th century by Ebelman[305,306] and Graham[307], the sol-gel process refers to an inorganic polymerization of molecular precursors to form an oxide network at low temperatures and under mild conditions. It is characterized by a transition from a sol (stable suspension of colloidal particles within a liquid) to a gel (3-dimensionally interconnected solid network holding large amounts of solvent). Precursors are generally metal alkoxides among which silicon alkoxides, Si(OR)₄, are the most commonly used. One strength of the sol-gel chemistry resides in its compatibility with many processing methods (e.g. spinning, dip coating, casting, spray-drying) that lead to a wide range of products: fibers, coatings, films, xerogels, aerogels, nanoparticles, etc. It enables the preparation of advanced tailor-made materials.[308] But its utmost attracting feature for the synthesis of materials containing fragile biomolecules is that the sol-gel process may proceed in water, at room temperature under mild conditions and in a very selective way (see 2.6.1). For this reason, it was given the label “Chimie douce” by Jacques Livage.[309] The silicon alkoxides react together without affecting other chemical functions present in biomolecules. In that respect, sol-gel may be considered a bio-orthogonal reaction. Interestingly, homogeneous multi-component systems can be easily accessed by simply mixing different molecular precursors. Sol-gel was thus used to synthesize numerous hybrid inorganic/organic and bioorganic materials but its potential as an inorganic cross-linking method for cell encapsulation in hydrogels has only recently been explored.

2.6.1. Mechanism of the sol-gel process: hydrolysis and condensation
The sol-gel process is based on two steps, hydrolysis and condensation. It starts with the hydrolysis of alkoxysilyl groups (Si-OR) into silanols (Si-OH) (Fig. 30, a). At neutral pH, this reaction is very slow (Fig. 31). However, acids, bases and nucleophiles can be used as catalysts to increase the reaction rate.

\[
\begin{align*}
\text{(a)} & \quad \text{(RO)}_3\text{Si-OR} + \text{H}_2\text{O} \rightarrow \text{(RO)}_2\text{Si-OH} + \text{R-OH} \\
\text{(b)} & \quad \text{(RO)}_3\text{Si-OH} + \text{(RO)}_3\text{Si-OH} \xrightarrow{\text{oxolation}} \text{(RO)}_3\text{Si-O-Si-(OR)}_3 + \text{H}_2\text{O} \\
& \quad \text{(RO)}_3\text{Si-OH} + \text{(RO)}_3\text{Si-OR} \xrightarrow{\text{alkoxolation}} \text{(RO)}_3\text{Si-O-Si-(OR)}_3 + \text{R-OH}
\end{align*}
\]

**Fig. 30.** (a) Overall equation of the hydrolysis of a silicon alkoxide into silanol. (b) Condensation reactions via oxolation or alkoxolation.
Fig. 31. Reaction rates of tetraethyl orthosilicate hydrolysis (blue curve) and condensation (green curve) as a function of pH.[310]

In the acid-catalyzed process, an oxygen from an alkoxysilyl group is protonated (Fig. 28, a). This protonation increases the electrophilicity of the silicon atom and favors the nucleophilic attack of water. Furthermore, it makes ROH a better leaving group. Both effects contribute to a high hydrolysis rate. Under basic conditions, hydroxyl ions add to the silicon center to yield a penta-coordinate silicon intermediate with a negative charge (Fig. 28, b) which makes the silicon center more nucleophilic.[311] The product of hydrolysis results from the release of an alcoholate. The overall reaction rate is lower than the acid-catalyzed reaction. Alternatively, nucleophile catalysts can be used to accelerate the process at neutral or physiological pH. Owing to their high affinity for silicon, fluorides hold a prime position as nucleophile catalysts for this reaction. The addition of a fluoride to a silicon atom produces a penta-coordinate silicon intermediate similar to the intermediate observed in the base-catalyzed reaction (Fig. 28, c). The penta-coordinate silicon is transformed into a hexa-coordinate silicon upon attack of a water molecule. The product of hydrolysis is formed upon release of an alcohol and regeneration of the fluoride catalyst.

Fig. 282. Acid- (a), base- (b) and fluoride- (c) catalyzed hydrolysis of a silicon alkoxide group.

The precursor may undergo four successive hydrolyses until Si(OH)$_4$ forms but as soon as one alkoxide group has been hydrolyzed, condensation can also start.
The condensation step leads to the formation of siloxane bonds (Si-O-Si). When the condensation reaction occurs between two hydroxysilyl groups with release of water, it is called oxolation (Fig. 30, b). When it involves a hydroxide and an alkoxide with release of an alcohol, it is called alkoxolation. Acids, bases and nucleophiles can also catalyze the condensation step. The reaction mechanism is exactly the same than the mechanism of hydrolysis except that silanols and silanolates are the nucleophilic species instead of H₂O and HO⁻ under acidic and basic conditions respectively. Likewise, in the presence of fluorides, a penta-coordinate silicon fluoride is formed and evolves towards a hexa-coordinate silicon center upon nucleophilic attack of a silanol. The condensation reaction ends with the release of H₂O (oxolation) or ROH (alkoxolation) and the regeneration of the fluoride ion.

2.6.2. Cell-encapsulation in hybrid hydrogels cross-linked by the sol-gel process

The encapsulation of cells in sol-gel materials started with the embedment of yeast cells [312,313] and bacteria [314–317] in SiO₂ hydrogels. Amorphous silica is considered non-toxic, being used commonly as an additive in food and drugs and recognized as safe (GRAS) by the American Food and Drug Administration.[318] Although it has also been demonstrated that animal cells encapsulated in sol-gel silica were viable,[313] in order to obtain a cell-friendly environment, it is important to keep the amount of silicon as low as possible to maximize the resemblance of the hydrogel with native ECM and limit the release of alcohol when alkoxide precursors are used. This can be achieved by using silicon atoms only as hydrogel cross-linkers. Precursors of such hydrogels are ‘hybrid’ molecules in the sense that they display both a (bio)organic moiety and inorganic alkoxysilane groups. They can be obtained by modifying covalently a (bio)polymer to introduce a trialkoxysilane moiety. In this case, the silicon atom is linked to the polymer via a C-Si bond. The network results from the formation of siloxane bonds (Fig. 3). Since the C-Si bond is hydrolytically stable, the network can be degraded either by hydrolysis of the siloxane bonds or by biodegradation of the backbone by pathways specific to each polymer. Depending on the chosen silylation reagent, the link between the polymer and the silylation reagent can also be specifically cleaved. Toxicity may arise from silylation reagents left unreacted in the hydrogel. To overcome this problem, it is possible to perform the modification of the polymer separately from the formation of the hydrogel. Washings between the two steps enable the elimination of free reagents and potential side-products.

Hybrid precursors are obtained by covalent modification of polymers with alkoxysilane moieties (Fig. 343). The choice of silylation reagent depends on the available reactive functions (e.g. alcohol, amine, carboxylic acid) displayed by the polymer (Fig. 34). Jo and Park described the functionalization of the hydroxyl extremities of PEG and Pluronic F-127 (block copolymer HO-[CH₂CH₂O]ₙ-[CH(CH₃)CH₂O]ₘ-[CH₂CH₂O]ₙ-H with n ≈ 98 and m ≈ 67) with 3-isocyanatopropyl triethoxysilane (ICPTES), but they used a sol-gel procedure involving ethanol, acidic pH and solvent evaporation.[127] Our group proposed a different procedure to prepare hydrogels from ICPTES-modified PEG.[126] The process was catalyzed by fluoride and carried out at physiological pH in a phosphate buffer at 37°C. Although the encapsulation of cells in these hydrogels during their
formation was not investigated, this procedure was cell-friendlier than the one described by Jo and Park.

![Chemical structures](image)

**Fig. 34.** Functionalization of polymers with trialkoxysilylated reagents.

Silylet biopolymers such as gelatin[128,137,319,320], chitosan[131,132], collagen[133] or even alginate[134] were already used to prepare hydrogels. However, they were prepared in non-biocompatible conditions and extensively washed after gelation or freeze-dried and re-swelled before being cell-seeded. Shirosaki et al. added GPTMS to chitosan in 0.25 M acetic acid aqueous solution. These conditions allowed both the covalent modification of chitosan with a trimethoxysilane group and the acid-catalyzed hydrolysis of methoxy groups into hydroxyl groups. Under such acidic conditions, condensation was quite slow. Therefore, the solution was neutralized with sodium hydroxide to promote condensation. At this stage, the hybrid chitosan solution was injectable. A 2 wt% chitosan solution could turn into a gel at 37°C within few hours. This hydrogel was intended to be used as a resorbable vehicle for bone graft; however, even though it is biocompatible, it does not encapsulate cells.[130] Finally, Weiss and co-workers developed hybrid cellulose-based hydrogels. They functionalized different cellulose derivatives, in particular hydroxypropylmethylcellulose (HPMC), with either GPTMS or 3-glycidopropylmethyldiethoxysilane (GPMDS) via a Williamson reaction between the hydroxyl groups of the biopolymer and the epoxide.[321,322] In organic solvents, the cellulose derivatives were not soluble and the reaction occurred in heterogeneous medium. In order to increase modification homogeneity, these authors developed and patented a silylation method in homogeneous medium by using ionic liquids.[323] Their sol-gel procedure consisted of a hydrolysis step at pH > 12.3 and condensation upon neutralization. At high pH, the hybrid polymer, named Si-HPMC, is in sodium silanolate form. The charge repulsion prevents condensation and allows the storage of the solution as a viscous liquid for a prolonged period. Condensation occurs upon neutralization at room or body temperature. They demonstrated that Si-HPMC hydrogels could encapsulate chondrocytes and be injected for cartilage tissue repair.[135] Eventually, they used Si-HPMC as a base for the development of several materials for tissue regeneration.[140,139,324,137,325,138,141]

Recently, our group proposed a synthetic hydrogel obtained in one step by sol-gel polymerization of a silylated nonapeptide derived from the consensus collagen sequence [Pro-Hyp-Gly]. Three tripeptide repeats were used and flanked by two lysine residues whose side chains were modified by triethoxysilane groups (Fig. ). The size of the hybrid peptide was too small to expect formation of a triple helix, but long enough to adopt a well-defined PPII structure.[326] Sodium fluoride was used as a catalyst to induce both the condensation and the hydrolysis process, at physiological pH and
37°C. Mesenchymal stem cells (MSC) were poured into the solution before gelation. Their viability was fully conserved after more than 24 hours, demonstrating that the short hybrid peptide was able to provide a cell-friendly environment. More recently, a silylated-HMPC based hydrogel obtained from an isolated silylated biopolymer precursor, was also used for direct cell encapsulation and allowed the extrusion-based 3D-bioprinting of stem cells with a good viability (T. Montheil et al., Sol-gel bioprinting, publication under consideration).

![Fig. 35. Bisilylated hybrid peptide as precursor for a cell-containing, collagen-inspired chemical hydrogel.](image)

### 3. Conclusions and future direction

Cell therapy is becoming one of the treatments of choice to repair damaged tissues, treat enzyme and hormone deficiencies, and fight cancers. Further developments in the field require the design of customized artificial biomimetic matrices providing cells adequate support but also communicating to them the signals they need to behave as required. So far, considerable efforts have been made to develop hydrogel scaffolds addressing a particular health issue. The development of more universal systems (i.e. applicable to each type of tissue) would be a significant breakthrough. To build up the hydrogel network with good control over the resulting physicochemical properties (density, biodegradability, hydrophilic/hydrophobic balance, etc.) and, at the same time, to decorate it with
various cell-stimulating molecules, each starting building block has to be chemically modified with one or several ‘clickable’ moieties. This is where the greatest challenge for the chemist probably lies: the assembly process must preserve cell viability upon encapsulation, it should be simple enough to allow the combination of different building blocks at a chosen ratio and it should proceed, if possible, in a single or limited number of steps. In addition, chemistries compatible with different shaping procedures would be an asset. Indeed, injection of cell-laden hydrogels at the site of interest constitutes a minimally invasive treatment while 3D printing enables access to structurally complex scaffolds and in vivo cell delivery in a well-organized manner. The easy preparation of bioinks sharing the same reticulation chemistry but displaying different compositions in bioactive components would simplify the reconstitution of spatially non-homogeneous tissues.

Acknowledgements
C. Echalier’s PhD was partly funded by the “Region Languedoc Roussillon”, grant attributed to G. Subra, through the program ‘Chercheur d’Avenir’.
L. Valot’s PhD was funded by the ANR (Agence Nationale de la Recherche), the French National Research Agency (ANR-16-CE18-0003).

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