NEW APPROACHES IN HYDROGEL SYNTHESIS – CLICK CHEMISTRY: A REVIEW

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Received September 12, 2011

Hydrogels have attracted great attention over the past decades, since they can be used for a variety of applications, including drug delivery systems and scaffolds for tissue engineering and repair. "Click chemistry", in particular copper (I)-catalyzed azide-alkyne cycloaddition (CuAAC), has been widely used in the preparation of complex architectures, such as hydrogel networks, due to its reaction specificity, quantitative yields and good functional group tolerance. The aim of this review is to present the synthesis and further use of "click" hydrogels composed of primarily natural components, using Cu as a catalyst or, following the latest trends, *via* a copper-free method.

Keywords: "click chemistry", polysaccharide, crosslinking, hydrogel, catalyst-free

INTRODUCTION

Hydrogels, "cross-linked macromolecular networks swollen in water or in biological fluids",[1] have received extraordinary attention as biomaterials used for biomedical and pharmaceutical applications. Of the numerous polymers that have been proposed for the preparation of hydrogels, polysaccharides have a number of advantages over synthetic polymers, which were initially employed in the field of pharmaceuticals. Generally, polysaccharides are non-toxic, biocompatible, biodegradable, abundant and susceptible of enzymatic digestion in the body. Biodegradability is especially useful for the release of drugs at a certain time and/or at a certain site in the body.

In recent years, a particular category of hydrogels has received extensive attention: the *in situ* formed gels, as they are useful in minimally invasive applications, as well as in drug delivery systems in which the sol-gel transition aids the performance of the formulation. The approaches for *in situ* gelling chemistry necessarily require: 1) chemical stability in water and in air; 2) non-toxic functional groups and crosslinking reactions; 3) rapid and controllable crosslinking kinetics. Also, preferably, the crosslinking moieties should be unreactive with the other functional groups that may be present during the application.

A concept that meets the above-mentioned standards is undoubtedly the 1,3-dipolar cycloaddition of an organic azide to an alkyne, which has demonstrated its potential in hydrogel networks,[2] being stable under physiological conditions. This reaction typically requires the use of a copper (I) catalyst, with known toxicity.[3-5] To make these hydrogels more biocompatible, prior to their utilization in biomedical fields, the Cu(I) catalyst must be leached out of the gel.[6] On the other hand, catalyst-free "click chemistry" is an underexplored crosslinking approach for generating non-toxic, bioorthogonal hydrogel networks *in situ*.

The present review, covering the literature of the last 5 years, focuses on the synthesis of these macromolecular architectures by an elegant utilization of "click chemistry", either with or without catalyst. Their applications in biomedical fields are also mentioned.

Crosslinking *via* CuAAC

The first illustration of hydrogel synthesis using "click chemistry" was made by Hilborn and co-workers[7] in 2006. They synthesized poly(vinyl alcohols) (PVA) functionalized with either acetylene or azide groups. Hydrogel was formed in a few minutes after the addition of $CuSO_4$/Na ascorbate.

Two years later,[8] they reported the first crosslinking of hyaluronic acid by means of a polymeric multifunctional crosslinker, such as PVA, as well as new crosslinking chemistries, leading to the formation of oxime, semicarbazone and thiazolidine crosslinks for the production of hyaluronan-based hydrogel materials. The synthesized PVA-based multifunctional crosslinkers showed fairly good cytocompatibility, exhibiting no signs of cytotoxicity after incubation for up to 48 h at the concentration normally used to crosslink aldehyde-modified hyaluronic acid (the maximum possible concentration of a free gel forming a PVA component, which could be expected in the case of no gel formation at all). The prepared HA/PVA hydrogels presented a wide range of mechanical properties, being formed in less than 1 min, at pH 7.4 and 37 °C, and might provide *in vivo* injectable materials for different biomedical applications.

The *in vivo* application field was also investigated by Chawla *et al*.,[9] who developed saccharide-peptide hydrogels as new synthetic extracellular matrices for regenerative medicine applications. This was the first study to use the Michael-type addition (Fig. 1) to form biodegradable synthetic hydrogels primarily composed of natural building blocks without a large synthetic component, such as PEG.

They applied the copolymerization of cysteine (Cys) and vinyl sulfone (VS)-functionalized saccharide-peptide polymers *via* Michael-type addition, for the encapsulation and 3D culture of mesenchymal stem cells (MSCs). The mechanical properties of the hydrogels were tunable by varying the VS:Cys ratio, as well as the pH of the crosslinking components. At the same time, the pH of crosslinking and the VS:Cys ratio have also influenced the degradation behavior of VS:Cys gels, with basic pH (pH = 8), which resulted in an accelerated loss of mass.

Compared to other hydrogel systems, the saccharide-peptide hydrogels described offer one of the most important advantages, namely their natural composition, which makes them eventually degradable into natural, non-toxic and bioabsorbable metabolites. Adult bone-marrow-derived mesenchymal stem cells (MSCs) may be obtained from a patient, then isolated and culture-expanded *in vitro*. Eventually, these 3D constructs may be transferred *in vivo* as implantable tissues for the treatment of diseases or injuries.

Another field investigated by many researchers consists in the creation of supports for cell culture and proliferation.

Another biodegradable PEG–peptide hydrogel has been synthesized using "click chemistry".[10] A series of Arg-Gly-Asp (RGD) containing peptides was prepared *via* the solid phase synthesis approach, then further functionalized with azide to yield peptide azide or peptide diazide. A tetra-hydroxy terminated 4-arm PEG was functionalized with acetylene and reacted with peptide azide/diazide and/or PEG diazide, to produce hydrogels *via* CuAAC. The gelation time ranged from 2 to 30 min, depending on temperature, catalyst and precursor concentration, as well as on peptide structure. Hydrogels crosslinked by peptide diazide yielded higher storage modulus (G0) with shorter spacers between the azide groups. The swelling degree decreased while G0 increased with increasing the concentration of the precursors, as a result of increased crosslinking density. Primary human dermal fibroblasts were used as model cells to explore the possibility of using RGD peptide hydrogels for cell-based wound healing. It was noticed that the RGD peptide hydrogels synthesized at a peptide concentration of 2.7-5.4 mM achieved significantly improved cell attachment and a higher cell proliferation rate, comparatively with hydrogels without RGD peptides.

Along the same line, Gao *et al.*[11] discussed the synthesis of a hydrogel obtained from hyaluronic acid (HA), chondroitin sulfate (CS) and gelatin *via* "click chemistry", in order to mimic the natural cartilage extracellular matrix. HA and CS were modified with 11-azido-3,6,9-trioxaundecan-1-amine (AA), and gelatin was modified with propiolic acid (PA). After mixing HA–AA, CS–AA and G–PA in the presence of

Cu (I) as catalyst, at a concentration of 0.95 mg mL^{-1}, the biological hydrogel (Fig. 2) was formed at room temperature, within 5 min. *In vitro* cell culture confirmed that chondrocytes could adhere and proliferate on the material.

Simultaneous interpenetrating polymer networks (sIPNs), with applications as biomaterials for contact lenses, biomedical materials, artificial organs and drug delivery systems, were first prepared *via* simultaneous "click chemistry" and ATRP, from a mixture of poly(ethylene glycol)-diazide (N3-PEG-N3, Mn = 4000 g/mol), tetrakis(2-propynyloxymethyl)methane (TPOM), ethyl-2-bromobutyrate (EBB), CuBr, pentamethyldiethylenetriamine (PMDETA), and 2-hydroxyethyl methacrylate (HEMA) in dimethylformamide (Fig. 3).

These hydrogels exhibit a fast gelation rate and high gel yield, high swelling ratios and good mechanical and antifouling properties. The authors[12] showed that an increased amount of Cu(I) catalyst can reduce the gelation time, so that, by increasing the concentration of CuBr from 20 to 100% (relative to the mole concentration of azido and alkynyl groups), the gelation time decreases from about 38 to 2 min and from about 42 to 2 min, respectively.

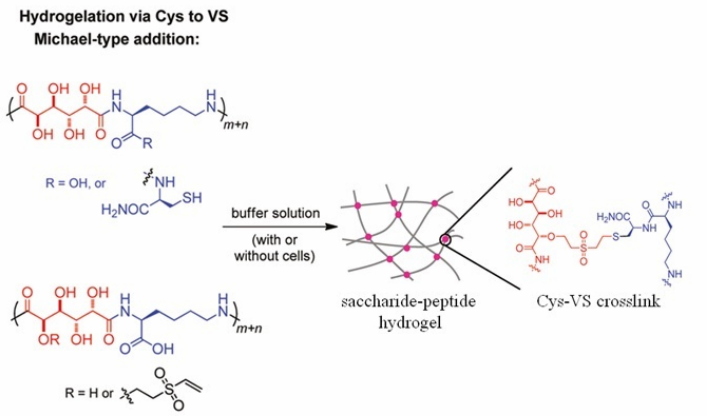

Figure 1: Hydrogel synthesis *via* vinyl sulfone-thiol conjugated addition, Chawla *et al.*[9]

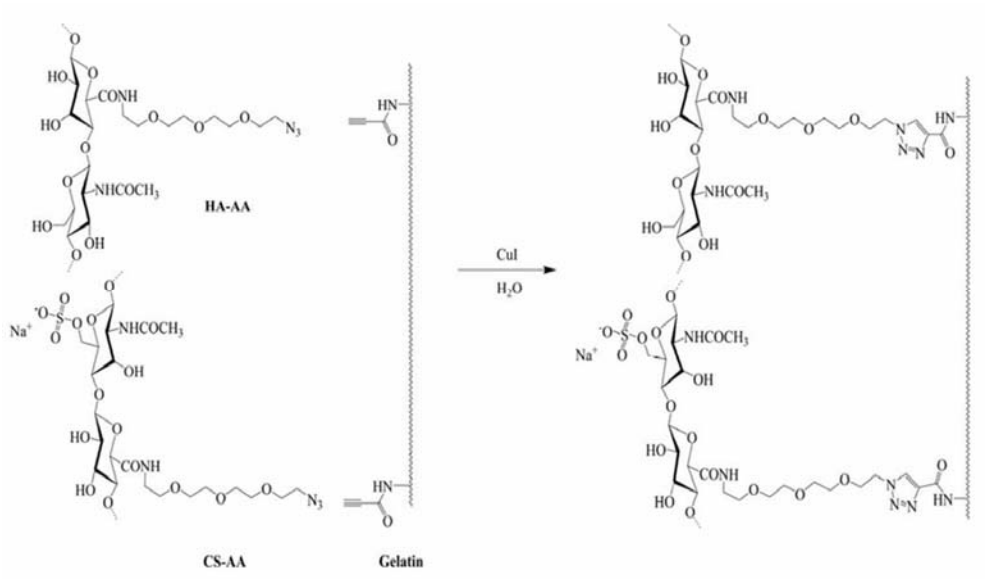

Figure 2: Synthesis of biological hydrogel based on hyaluronic acid (HA), chondroitin sulfate (CS) and gelatin *via* "click chemistry", Gao *et al.*[11]

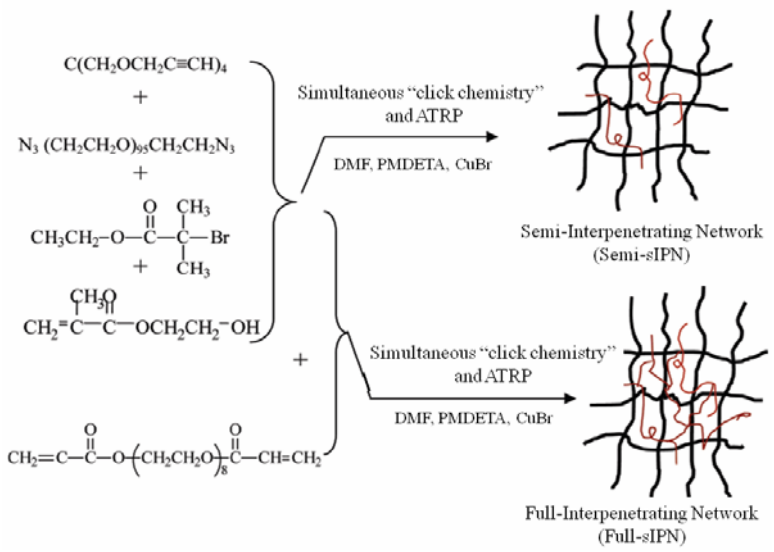

Figure 3: Preparation of semi-PEG/PHEMA-sIPN and full-PEG/PHEMA-sIPN *via* simultaneous "click chemistry" and ATRP, Xu *et al.*[12]

Another semi-interpenetrating polymer network with unique molecular structures – the PEG network with movable sliding-grafted poly(2-hydroxyethyl methacrylate) (PHEMA) (s-IPN-PEG/R-CD-sg-PHEMA) – has been reported.[13] These hydrogels were prepared by simultaneous "click chemistry" and atom transfer radical polymerization (ATRP) of a mixture of the poly(ethylene glycol)-diazide/bromo-butyryloxy R-cyclodextrin inclusion complex (N_3-PEGN_3/(R-CD-BIBB)$_m$), tetrakis(2-propy-nyloxymethyl) methane (TMOP), CuBr, pentamethyldiethylenetriamine (PMDETA), HEMA and DMF (Fig. 4).

The length of the sliding-grafted PHEMA of s-IPN-PEG/R-CD-sg-PHEMA can be regulated by changing the polymerization times. The s-IPN-PEG/R-CD-sg-PHEMAs exhibit good physical and mechanical properties. Most important, compared to classical semi-IPN, the diffusion of interpenetrated PHEMA from s-IPN-PEG/R-CD-sg-PHEMA was largely prevented for a long-time solvent immersion, as PHEMA brushes were fixed on the PEG networks. The sliding-grafted PHEMA chains afford functionalities to the bulk and surface of s-IPN-PEG and could be potentially used as carriers of genes and drugs.

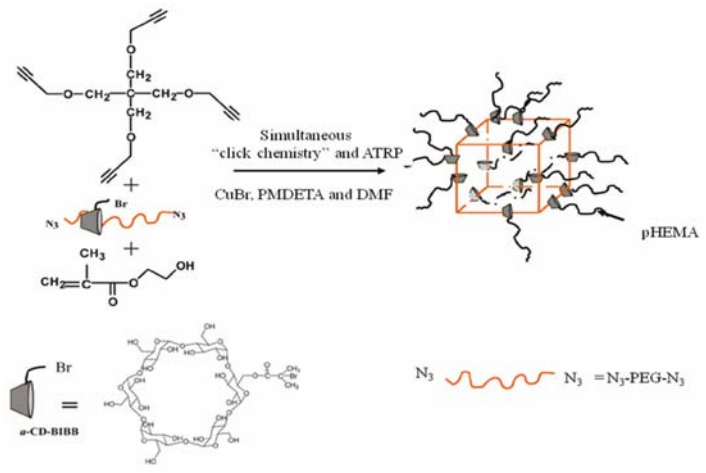

Figure 4: Preparation of PEG network with movable sliding-graft PHEMA *via* simultaneous ATRP and CuAAC, Yao *et al.*[13]

Figure 5: Hyaluronic acid hydrogel synthesis *via* Diels-Alder "click" reaction, Nimmo *et al.*[14]

For designing a simpler one-step, aqueous-based crosslinking system, Nimmo *et al.*[14] synthesized hyaluronic acid (HA) hydrogels *via* Diels-Alder "click chemistry" (Fig. 5). Furan-modified HA derivatives were synthesized and crosslinked *via* dimaleimide poly(ethylene glycol), in the absence of additional crosslinking agents or catalysts. By controlling the furan-to-maleimide molar ratio, both the mechanical and degradation properties of the resulting Diels-Alder hydrogels can be tuned.

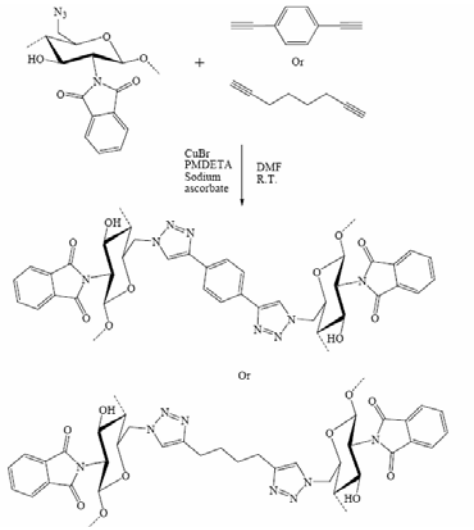

Figure 6: Synthesis of chitosan-based hydrogel, Zampano *et al.*[15]

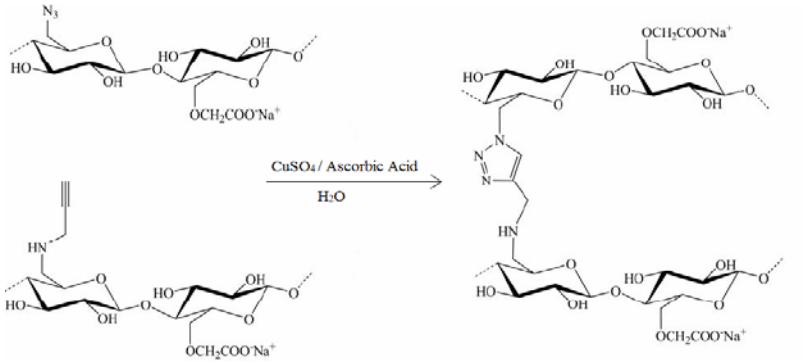

Figure 7: Synthesis of cellulose-based hydrogel, Heinze *et al.*[16]

Rheological and degradation studies demonstrate that the Diels-Alder "click" reaction is a suitable crosslinking method for HA. The HA-PEG hydrogels had an elastic modulus similar to that of the central nervous system tissue, demonstrating minimal swelling and complete degradation. Together with their cell-interactive properties, these HA-PEG hydrogels may be suitable for soft tissue engineering and regenerative medicine applications.

The workgroups of Zampano, Heinze and Argyropoulos have also taken advantage of the CuAAC versatility to obtain polysaccharide-based networks for biomedical applications. Therefore, Zampano *et al.*[15] synthesized a gel from 6-deoxy-6-azido chitosan, using an aliphatic or an aromatic chain bearing two alkyne functions (Fig. 6).

The azido products reacted with model reagent phenylacetylenes, the di-alkynes 1,7-octadiyne and 1,4-diethynylbenzene used for crosslinking *via* a "click" reaction at room temperature. All alkynes reacted efficiently both in heterogeneous and homogeneous phases.

Heinze *et al.*[16] obtained a novel cellulose-based hydrogel by preparing cellulose derivatives bearing azide and alkyne moieties (Fig. 7). First, they converted cellulose *p*-toluenesulphonic acid ester with sodium azide, after which they obtained propargylamine.

The products were carboxymethylated to yield water-soluble multifunctional cellulose derivatives, and CuAAC was applied for crosslinking. Gel formation occurred within 55 and 1600 s after mixing the aqueous solutions with both compounds, the catalytic system and the gelation time depending on the degree of functionalization and amount of copper (I) catalyst. The gels contained up to 98.4% water. Freeze-drying led to spongy materials with a porous structure.

The formation of nanoplatelet gel-like nanomaterials from cellulose nanocrystals (CNC) as starting materials was described by Argyropoulos *et al*.[17] Initially, the primary hydroxyl groups on the CNC surfaces were selectively activated to carboxylic acids by TEMPO-mediated hypohalite oxidation. In the next step, 11-azido-3,6,9-trioxaundecan-1-amine was grafted onto the activated oxidized CNC surface, *via* an amidation reaction. The grafted amine compounds presented terminal alkyne or azide functionalities and two sets of precursors were prepared. The alkyne and azide surface-functionalized CNC precursors were then brought together using "click chemistry", creating – for the first time – unique nanoplatelet gels (Fig. 8).

Thermoreversible hydrogels are promising matrices for tissue-engineered cartilages and spine constructs. They require specific properties during all stages of cell therapy (*e.g*., cell expansion, recovery, injection, delivery).

In 2009, Zhang *et al*.[18] reported the synthesis (Fig. 9) of a thermosensitive hydrogel obtained *via* Huisgen's 1,3-dipolar cycloaddition between azide-modified cellulose and an alkyne-modified P (NIPAAm-co-HEMA). The resulting material exhibited a porous network and temperature dependence of swelling ratio and reswelling kinetics. The authors mentioned that the strategy described would present a great potential for *in situ* formation of hydrogels from natural polysaccharides.

Mortisen *et al.*[19] have recently presented a semi-synthetic, thermoreversible hyaluronan-poly(*N*-isopropylacrylamide) hydrogel (Fig. 10) with well-defined molecular architecture and properties, synthesized through RAFT polymerization and "click chemistry".

They showed that the efficiency of the "click" reaction facilitates control of the substitution degree of PNIPAM chains and that RAFT polymerization allows the preparation of PNIPAM with controlled molecular weight and low PDI. The control of the critical parameters of PNIPAM molecular weight and grafting density allowed the optimization of the gel for regenerative medicine applications, where the expansion of autologous cells is required prior to delivery to an injured site. Therefore, a 3D matrix with reversible gelling for encapsulation of cells has to be produced, which will allow the retrieval and further manipulation of *in vitro* cultured cells. It has been found out that reversibility of the PNIPAM gelling process improved in the presence of HA, however, a higher grafting density led to lower mechanical properties. All hydrogels and their degradation products were cytocompatible with hTERT-BJ1 fibroblasts.

Tizotti *et al*.[20] developed a new thermosensitive guar-based hydrogel by crosslinking alkyne-functionalized guar chains and α,ω-diazido poly[(ethylene glycol)-co-(propylene glycol)] (PEG-co-PPG) through CuAAC coupling (Fig. 11).

The reaction was successfully performed in water, under mild conditions, at relatively shorter

gelation times. It was found out that both mechanical and swelling properties can be efficiently tuned by varying the experimental parameters, such as the CuAAC coupling temperature, guar molecular weight and mass fraction of the guar/crosslinker chains. It appears that, at a sufficient poly(oxyalkylene) crosslinker content (70 wt%), the resulting hydrogel shrinks while heating. The preliminary data available on the capability of these networks to act as containers for the temperature-controlled delivery of doxorubicin proved to be easily adjustable by changing the amount of PEG-co-PPG crosslinker in the network, owing to the thermo-dependent microstructure of hydrogels.

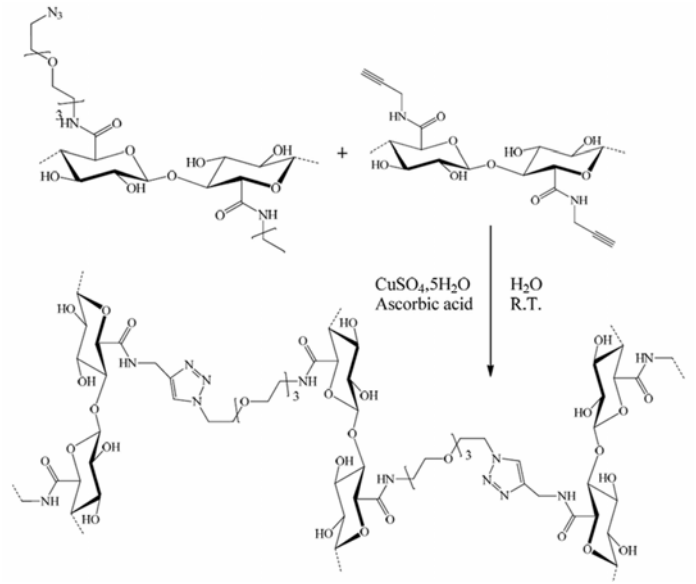

Figure 8: Synthesis of hydrogel nanomaterials from cellulose nanocrystals, Argyropoulos *et al.*[17]

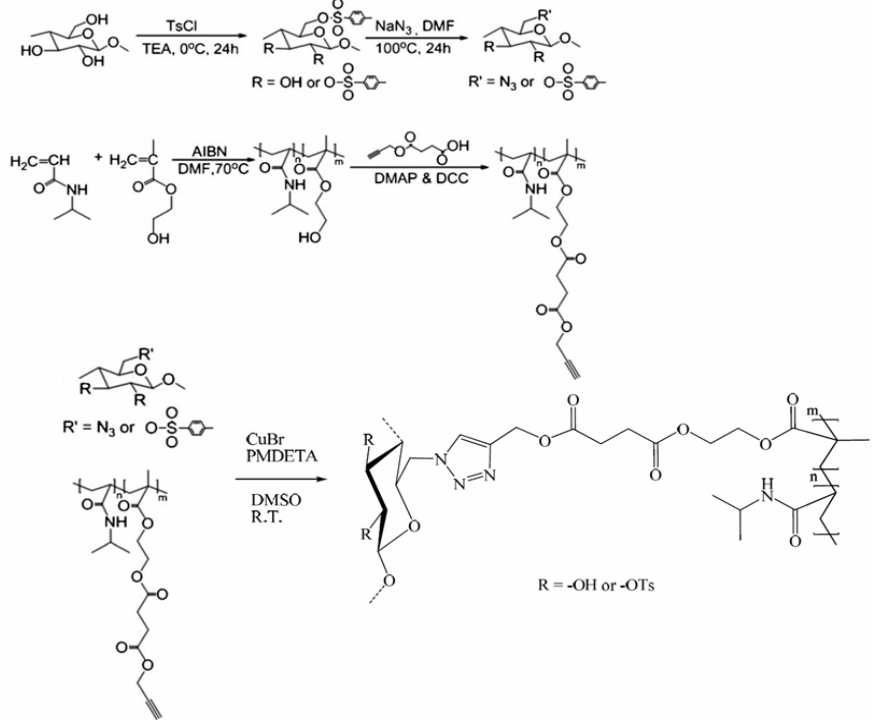

Figure 9: Thermosensitive hydrogel from azide-modified cellulose and an alkyne-modified

P(NIPAAm-*co*-HEMA), Zhang *et al.*[18]

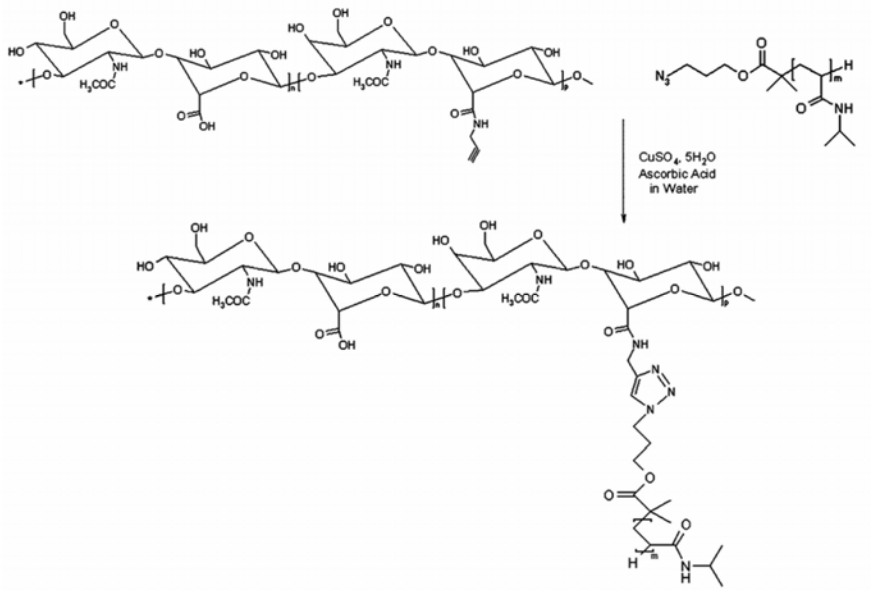

Figure 10: CuAAC of hyaluronan propargylamide (HApa) with azido-terminated poly(N-isopropylacrylamide), Mortinsen *et al.*[19]

Malkoch *et al.*[21] described the synthesis of well-defined PEG-based hydrogels using a combination of α,ω-alkyne-telechelic linear PEG chains of various molecular weight and azido-tetrafunctional short PEG crosslinkers. Network formation was reported to take place in less than 30 min, under standard CuAAC conditions, and in less than 1 min, when subjected to microwave irradiation. These "click" hydrogels exhibited much higher swelling capacities and improved mechanical properties, as compared to the standard photochemically crosslinked hydrogels.

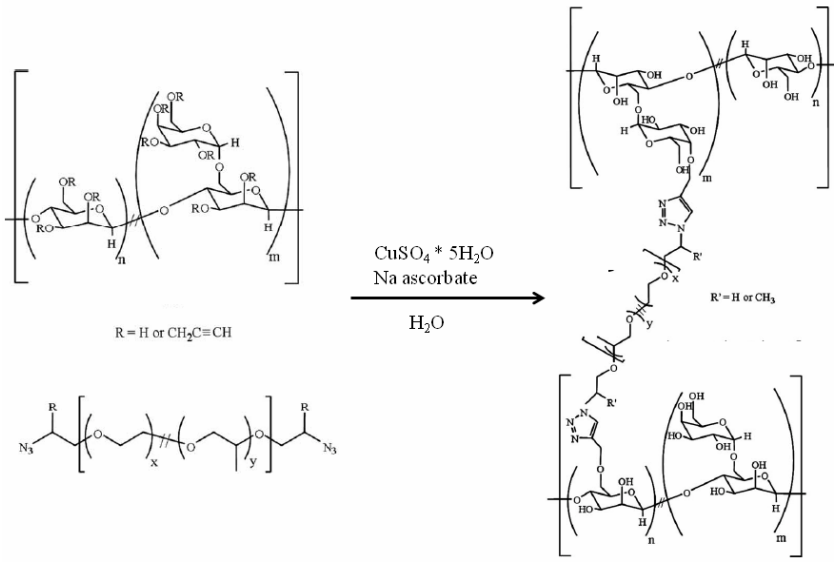

Figure 11: Synthesis of guar-based hydrogels, Tizotti *et al.*[20]

For example, a comparison of gels made from PEG with molecular weights around 10 kDa showed that the click-gels extended to 1550% of their original dimensions before breaking, *i.e.* 10

times higher than those of the acrylate-based gel. Moreover, the high chemoselectivity of CuAAC allowed the preparation of these PEG-hydrogels in the presence of various functional fillers, without affecting adversely the extent of cross-linking. The authors also demonstrated the facile removal of copper, to allow the use of hydrogels in biological systems.

In another example of incorporating functional moieties into a network, Lecomte[22] described the synthesis of biodegradable amphiphilic networks with pH responsive properties, by a combination of ROP and CuAAC crosslinking. The ROP of R-chloro-ε-caprolactone, followed by azide displacement, was used to produce poly(R-azido-ε-caprolactone). Before being used to crosslink dialkynyl PEG, the poly(caprolactone) sample was functionalized with *N*,*N*-dimethylprop-2-yn-1-amine along the backbone, in various loadings, using CuAAC. The crosslinking reaction was performed in the same reaction vessel, by simply adding the functional PEG to the reactor and allowing gelation to occur. The resulting gels were amphiphilic in nature, and the pH-dependent release of a guest was demonstrated using a model dye.

A novel procedure for the *in situ* rapid chemical gelation was employed by Crescenzi *et al.*,[23] to produce hyaluronan (HA) hydrogels (Fig. 12) and to encapsulate yeast cells during crosslinking.

Water-soluble polysaccharide derivatives bearing side chains endowed with either azide or alkyne terminal functionality have been prepared and hydrogels were formed within a few minutes, by dissolving both components in H_2O in the presence of 1% w/v of CuCl, at room temperature. The amount of Cu(I) used was non-toxic for both yeast cells and red blood cells, included in the same way into HA-based click-gels. After 24 h, about 60% of the entrapped cells exhibited proliferating activity. Also, the synthesized hydrogels were evaluated for drug-release capabilities with doxorubicin and benzidamine as model drugs. Benzidamine was quantitatively released within hours, whereas the release of doxorubicin was prolonged over several weeks, the slower release of doxorubicin being explained by the strong electrostatic interactions between the protonated amino group of doxorubicin and the carboxylate groups of hydrogels.

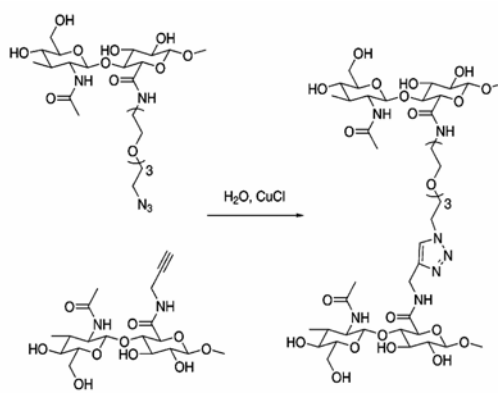

Figure 12: Synthesis of HA-based click-gels, Crescenzi *et al.*[23]

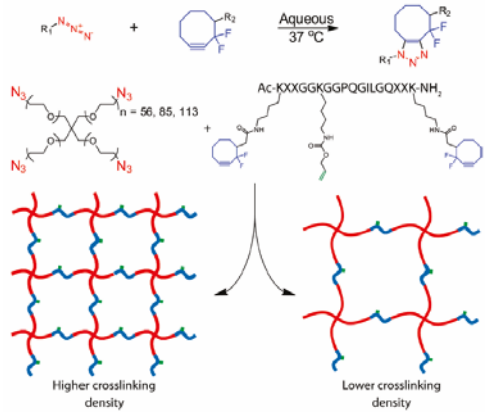

Figure 13: Synthesis of click-hydrogel from tetrafunctional poly(ethylene glycol) molecules and difunctionalized synthetic polypeptides, DeForest *et al.*[27]

Crosslinking *via* copper-free cycloaddition

Even though the "click" reaction has been intensively exploited in many fields, one of its important limitations is the toxicity of the catalytic system and the difficulty to implement this approach in biological applications – it is very difficult to ensure that all copper has been removed from the gels even after intensive extraction. Such copper drawbacks of CuAAC crosslinking can be overcome by using a copper-free option that utilizes fluorinated cyclo-octyne reagents. This reaction,[24] strain-promoted azide–alkyne cycloaddition, has been shown to proceed very efficiently with high chemoselectivity, even for *in vivo* applications, making it perfectly suitable for *in situ* crosslinking.

Hence, direct encapsulation of cells in a clicked PEG-peptide hydrogel was achieved using

macromolecular alkyne and azide precursors, in combination with a copper-free difluorocyclooctyne (DIFO3) "click chemistry".[25] This cyclo-octyne has a ring strain and electron-withdrawing fluorine substituents that give rise to an activated alkyne, which reacts quickly with azides, hence promoting dipolar cyclo-addition, without using a catalyst.[26] A four-arm poly(ethylene glycol) (PEG) tetra-azide was reacted with bis(DIFO3) di-functionalized polypeptide in an aqueous environment, at 37 °C.

DeForest's[27] work of 2010 illustrates a strategy where step-growth networks are rapidly formed *via* a copper-free, azide-alkyne "click chemistry" between tetrafunctional poly(ethylene glycol) molecules and di-functionalized synthetic polypeptides with alkyne groups (Fig. 13).

The molecular weight of the polymer precursors (10, 15, or 20 kDa PEG) and the stoichiometry of the reactive end group functionalities (1.5:1 to 1:1.5) provide control over the material crosslinking density, enabling elastic materials with tunable moduli. A sequential photo-chemically activated thiol-ene chemistry allows subsequent functionalization of the network through a reaction with pendant alkyne moieties on the peptide. As the thiol-ene reaction is light-driven, the degree of modification is directly related to the dosage of light delivered to the system (0-6 Jcm^{-2}). Since both reactions can occur in the presence of cells, this material ultimately enables independent and *in situ* tuning of the biochemical and biomechanical properties of biomaterial networks, suggesting a path to direct cell function throughout specific regions within a 3D material.

Crosslinking of water-soluble azide-functionalized polymers has been demonstrated with an electron-deficient dialkyne crosslinker, to form hydrogels at physiological temperature, without addition of copper (I) catalyst. The authors[28] noted that the fastest gels obtained during their study crosslink too slowly to be used in most *in situ* applications. It is possible, however, to improve the gelation kinetics by modifying crosslinking moieties (*e.g*. alkyne with increased electron deficiency, azide with increased electron density) and thus the reaction rate.

Li *et al*.[29] demonstrated the efficient formation of small molecule azides, including a DNA-immobilized azide, with various small molecule electron-deficient alkynes in water, at room temperature. Even if the reactions with a bulkier DNA-immobilized azide generated lower yields (45-67%) at increased reaction times (48 h), yields were noted to be as high as 94% within 6-12 h. They reported the first use of Cu (I)-free "click chemistry" *via* electron-deficient di-alkyne crosslinkers with azide-functionalized polymers, for hydrogel formation under physiological conditions.

CONCLUSIONS

Approximately 50 years ago, Lím[30] invented hydrogels. Since then, the evolution of the design of new macromolecular drug delivery systems and biomaterials has been astonishing.

The works presented in the previous paragraphs illustrates the last 5 years' impact of Sharpless' "click chemistry" on the synthesis of hydrogels and their future applications, demonstrating that this approach can afford classic, *in situ* and copper-free gelation with well-defined properties.

Even if CuAAC requires a cytotoxic catalyst, this reaction has been shown as highly suitable for hydrogel synthesis, with future applications in drug delivery and tissue engineering fields.

With increasing efforts devoted to controlled molecule release, the applications of hydrogels will continue to grow in the future, with at least the same enthusiasm as today.

ACKNOWLEDGEMENTS: This study has been carried out with the support of BRAIN "Doctoral scholarships as an investment in intelligence" project, financed by the European Social Fund and the Romanian Government. Also, one of the authors (MD) acknowledges the financial support through the Romanian POSDRU/89/1.5/S/49944 project.

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