Video Article

The Synthesis of RGD-functionalized Hydrogels as a Tool for Therapeutic Applications

Emanuele Mauri[1], Alessandro Sacchetti[1], Filippo Rossi[1]

[1]Department of Chemistry, Materials and Chemical Engineering "Giulio Natta", Politecnico di Milano

Correspondence to: Filippo Rossi at filippo.rossi@polimi.it

URL: https://www.jove.com/video/54445
DOI: doi:10.3791/54445

Keywords: Bioengineering, Issue 116, Hydrogel, polyacrylic acid, RGD, click chemistry, microwave-assisted synthesis, functionalization, tissue engineering

Date Published: 10/7/2016

Citation: Mauri, E., Sacchetti, A., Rossi, F. The Synthesis of RGD-functionalized Hydrogels as a Tool for Therapeutic Applications. *J. Vis. Exp.* (116), e54445, doi:10.3791/54445 (2016).

Abstract

The use of polymers as biomaterials has provided significant advantages in therapeutic applications. In particular, the possibility to modify and functionalize polymer chains with compounds that are able to improve biocompatibility, mechanical properties, or cell viability allows the design of novel materials to meet new challenges in the biomedical field. With the polymer functionalization strategies, click chemistry is a powerful tool to improve cell-compatibility and drug delivery properties of polymeric devices. Similarly, the fundamental need of biomedicine to use sterile tools to avoid potential adverse-side effects, such as toxicity or contamination of the biological environment, gives rise to increasing interest in the microwave-assisted strategy.

The combination of click chemistry and the microwave-assisted method is suitable to produce biocompatible hydrogels with desired functionalities and improved performances in biomedical applications. This work aims to synthesize RGD-functionalized hydrogels. RGD (arginylglycylaspartic acid) is a tripeptide that can mimic cell adhesion proteins and bind to cell-surface receptors, creating a hospitable microenvironment for cells within the 3D polymeric network of the hydrogels. RGD functionalization occurs through Huisgen 1,3-dipolar cycloaddition. Some PAA carboxyl groups are modified with an alkyne moiety, whereas RGD is functionalized with azido acid as the terminal residue of the peptide sequence. Finally, both products are used in a copper catalyzed click reaction to permanently link the peptide to PAA. This modified polymer is used with carbomer, agarose and polyethylene glycol (PEG) to synthesize a hydrogel matrix. The 3D structure is formed due to an esterification reaction involving carboxyl groups from PAA and carbomer and hydroxyl groups from agarose and PEG through microwave-assisted polycondensation. The efficiency of the gelation mechanism ensures a high degree of RGD functionalization. In addition, the procedure to load therapeutic compounds or biological tools within this functionalized network is very simple and reproducible.

Video Link

The video component of this article can be found at https://www.jove.com/video/54445/

Introduction

Hydrogels are three-dimensional networks formed by hydrophilic cross-linked polymers, which are natural or synthetic, and characterized by a distinctive three-dimensional structure. These devices are increasingly attractive in the biomedical fields of drug delivery, tissue engineering, gene carriers and smart sensors[1,2]. Indeed, their high water content, as well as their rheological and mechanical properties make them suitable candidates to mimic soft tissue microenvironments and make them effective tools for water-soluble cytokine or growth factor delivery. One of the most promising use is as an injectable biomaterial carrying cells and bioactive compounds. Hydrogels may improve cell survival and control stem cell fate by holding and precisely delivering stem cell regulatory signals in a physiological relevant fashion, as observed in *in vitro* and in *in vivo* experiments[3,4]. The leading advantage of this is the possibility to maintain injected cells within the zone of inoculation (*in situ*), minimizing the amount of cells that leaves the area and extravasates into the circulatory torrent, migrating all over the body and losing the target goal[5]. The stability of the three-dimensional hydrogel networks is due to its cross-linking sites, formed by covalent bonds or cohesive forces among the polymer chains[6].

In this framework, orthogonal selective chemistry applied to polymer chains is a versatile tool able to improve hydrogel performances[7]. Indeed, the modification of polymers with suitable chemical groups could help to provide appropriate chemical, physical and mechanical properties to enhance cell viability and their use in tissue formation. In the same way, among the techniques to load cells or growth factors within the gel matrix, the use of the RGD peptide allows improvements in cell adhesion and survival. RGD is a tripeptide composed of arginine, glycine and aspartic acid, which is by far the most effective and often employed tripeptide due to its ability to address more than one cell adhesion receptor and its biological impact on cell anchoring, behavior and survival[8,9]. In this work, the synthesis of RGD-functionalized hydrogels is studied with the aim of designing networks characterized by sufficient biochemical properties for a hospitable cell microenvironment.

The use of microwave radiation in hydrogel synthesis offers a simple procedure to minimize side reactions and obtain higher reaction rates and yields in a shorter period of time compared to the conventional thermal processes[10]. This method does not require purification steps and yields

sterile hydrogels due to the interactions of the polymers and the absence of organic solvent in the reaction system[11]. Therefore, it ensures high percentages of RGD linked to the polymeric network because no modifications are required to the polymer chemical groups involved in gel formation. Carboxyl groups, from PAA and carbomer, and hydroxyl groups, from PEG and agarose, give rise to the hydrogel three-dimensional structure through a polycondensation reaction. The mentioned polymers are used for the synthesis of hydrogels in the spinal cord injury repair treatments[12]. These devices, as reported in previous works[13,14], show high biocompatibility as well as mechanical and physicochemical properties that resemble those of many living tissues and in thixotropic nature. Moreover, they remain localized *in situ*, at the zone of injection.

In this work, PAA carboxyl groups are modified with an alkyne moiety (**Figure 1**), and a RGD-azide compound is synthesized exploiting the reactivity of the tripeptide terminal group -NH_2 with a prepared chemical compound with structure $(CH_2)_n$-N_3 (**Figure 2**). Subsequently, the modified PAA reacts with the RGD-azide derivative through CuAAC click reaction[15-17] (**Figure 3**). The use of a copper(I) catalyst leads to major improvements in both the reaction rate and the regioselectivity. The CuAAC reaction is widely used in organic synthesis and in polymer science. It combines high efficiency and high tolerance to the functional groups, and it is unaffected by the use of organic solvents. A high selectivity, a fast reaction time and a simple purification procedure allow the obtainment of star polymers, block copolymers or chains grafting desired moieties[18]. This click strategy makes it possible to modify polymers after polymerization to customize the physicochemical properties according to the final biochemical application. The CuAAC experimental conditions are easily reproducible (the reaction is insensitive to water, whereas copper oxidation may occur minimally), and the nature of formed triazole ensures stability of the product. The use of copper metal can be considered a critical point, due to its potential toxic effect against cells and in the biological microenvironment, but dialysis is used as a purification method to allow the complete removal of catalytic residues. Finally, PAA modified RGD is used in hydrogel synthesis (**Figure 4**) and the physicochemical properties of the resulting networks are investigated, in order to check the potential functionality of these systems as cells or drugs carriers.

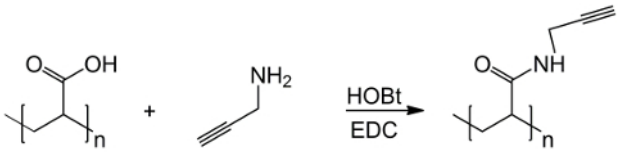

Figure 1: PAA modified alkyne synthesis. A scheme of PAA functionalization with alkyne group; "n" indicates the monomers with carboxyl group reacting with propargylamine. Please click here to view a larger version of this figure.

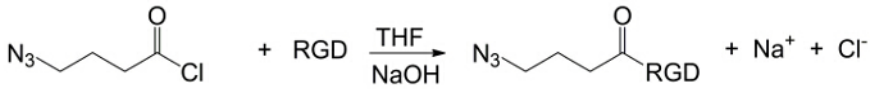

Figure 2: RGD-azide synthesis. The synthesis of RGD-azide derivative. Please click here to view a larger version of this figure.

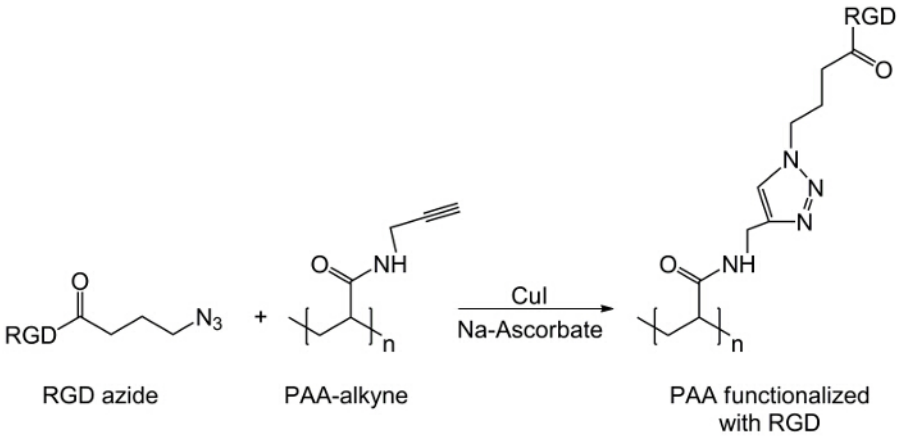

Figure 3: Click reaction. Scheme of click reaction between RGD-azide derivative and alkyne-PAA. Please click here to view a larger version of this figure.

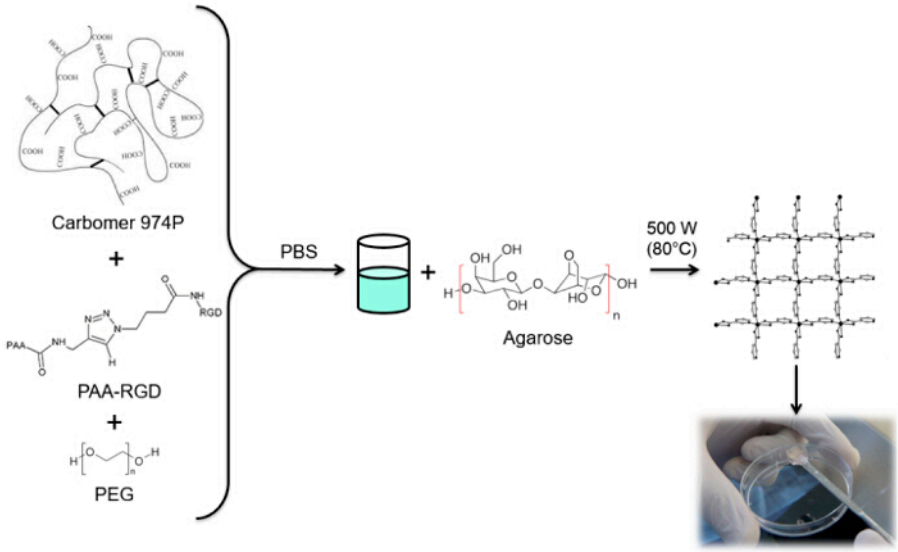

Figure 4: Hydrogel synthesis. RGD functionalized hydrogel synthesis procedure. Please click here to view a larger version of this figure.

Protocol

Note: The chemicals are used as received. Linear RGD is purchased, but it can be prepared by standard Fmoc solid phase peptide synthesis[16,19]. Solvents are of analytical grade. The dialysis requires the use of membrane with a M_w cut-off equal to 3,500 Da. The synthesized compounds are characterized by ^{1}H NMR spectra recorded on a 400 MHz spectrometer using chloroform ($CDCl_3$) or deuterium oxide (D_2O) as solvents, and chemical shifts are reported as δ values in parts per million. Furthermore, hydrogels are subjected to FT-IR analysis using KBr pellet technique and their physical characterization involves gelation studies assessed using the inverted test tube at 37 °C.

1. Synthesis of 4-Azidobutanoyl Chloride 1

1. Dissolve 500 mg of 4-azidobutanoic acid (3.90 mmol) in 10 ml of dichloromethane and 0.5 ml of dimethylformamide.
2. Cool the solution at 0 °C, using an ice bath.
3. Add 505 µl of oxalyl chloride (5.85 mmol) to 5 ml of dichloromethane and slowly add dropwise to the reaction system, while stirring.
4. After 1 hr at 0 °C using an ice bath, return to room temperature.
5. Remove the solvent under reduced pressure using a rotary evaporator.
6. Characterize the obtained product by ^{1}H-NMR spectroscopy, dissolving the sample in $CDCl_3$[16].

2. Synthesis of RGD-azide Derivative 2

1. Dissolve 50 mg of RGD (0.145 mmol) in 1 ml of 1 M NaOH.
2. Dissolve 24 mg of **1** (0.16 mmol) in 2 ml of tetrahydrofuran.
3. Add all of the RGD solution to solution **1** dropwise at 0 °C using an ice bath.
4. Return to room temperature and stir overnight.
5. Add 1 ml of 1 M HCl.
6. Remove the solvent under reduced pressure using a rotary evaporator.
7. Characterize the obtained product by ^{1}H-NMR spectroscopy, dissolving the sample in D_2O[16].

3. PAA Alkyne Modification 3

1. Dissolve 200 mg of 35% w/w PAA solution (2.8 mmol) in 15 ml of distilled water.
2. Add 15.4 mg of propargylamine hydrochloride (0.20 mmol).
3. Dissolve 42.8 mg of 1-hydroxybenzotriazole hydrate (HOBt, 0.28 mmol) in 14 ml of a 1:1 v/v acetonitrile:distilled water solution by heating to 50 °C.
4. Add all of the HOBt solution to PAA solution at room temperature.
5. Add 53.6 mg of ethyldimethylaminopropylcarbodiimide (EDC, 0.28 mmol) to the reaction mixture.
6. Use 1 M HCl to adjust the pH to 5.5 and stir the reaction system overnight at room temperature.
7. Dialyze the solution. Dissolve 11.2 g of sodium chloride in 2 L of distilled water and then add 0.2 ml of 37% w/w HCl. Dialyze the solution using a membrane with a M_w cut-off of 3.5 kDa.
8. Perform dialysis for three days. Change the dialysis solution daily with 2 L of freshly prepared distilled water containing 0.2 ml of 37% w/w HCl.
9. Store the final solution at -80 °C. Lyophilize it in a lyophilizer according to manufacturer's protocols.
10. Characterize the functionalized polymer by ^{1}H-NMR spectroscopy, dissolving the sample in D_2O[16].

4. Synthesis of PAA-RGD Polymer 4

1. Dissolve 78 mg of PAA modified alkyne **3** (1.083 mmol) in 10 ml of distilled water.
2. Dissolve 25 mg of the RGD azide **2** derivative (0.0722 mmol) in 5 ml of tetrahydrofuran.
3. Add all of the RGD solution to the polymeric solution.
4. Add 2.2 mg of copper iodide (0.0116 mmol) and 2.2 mg of sodium ascorbate (0.0111 mmol).
5. Reflux the resulting mixture overnight at 60 °C, with stirring.
6. Cool the mixture to 25 °C.
7. Dialyze the solution. Dissolve 11.2 g of sodium chloride in 2 L of distilled water and then add 0.2 ml of 37% w/w HCl. Dialyze the solution using a membrane with a M_w cut-off of 3.5 kDa.
8. Perform dialysis for three days. Change the dialysis solution daily with 2 L of freshly prepared distilled water containing 0.2 ml of 37% w/w HCl.
9. Store the final solution at -80 °C. Lyophilize it in a lyophilizer according to manufacturer's protocols.
10. Characterize the obtained product by ^{1}H-NMR spectroscopy, dissolving the sample in D_2O[16].

5. RGD-functionalized Hydrogel Synthesis

1. Prepare the PBS. Dissolve 645 mg of PBS salt in 50 ml of distilled water.
2. Blend 40 mg of carbomer and 10 mg of functionalized PAA 4 in 9 ml of PBS (step 5.1), at room temperature, until complete dissolution (30 min).
3. Add 400 mg of PEG to the solution and keep stirring for 45 min.
4. Stop the stirring and allow the system to settle for 30 min.
5. Use 1 N NaOH to adjust pH to 7.4.
6. To 5 ml of the obtained mixture, add 25 mg of agarose powder.
7. Irradiate the system with microwave radiation at 500 W until boiling, for a time usually between 30 sec and 1 min, and electromagnetically heat up to 80 °C.
8. Leave the mixture exposed to room temperature until its temperature decreases to 50 °C and add 5 ml of PBS (step 5.1), in order to obtain a solution at a 1:1 volumetric ratio.
9. Prepare 12 multiwell plate containing steel cylinders with a diameter of 1.1 cm.
10. Take 500 µl aliquots from the solution and place them to each steel cylinders.
11. Leave at rest for 45 min until complete gelification of the system.
12. Remove the cylinders using a stainless steel forceps to obtain the hydrogels.

6. Loading of Therapeutic Tool (Drug or Cells)

1. Repeat steps 5.1-5.7.
2. When the mixture (already at sol state) reaches 37 °C, add 5 ml of the solution containing the desired drug solution or cell culture, in order to obtain a final system at a 1:1 volumetric ratio.
3. Repeat steps 5.9-5.12 to obtain polymeric networks with biocompounds physically entrapped within the gel.

7. Hydrogel Characterization

1. FT-IR Analysis
    1. After gel formation, soak one of the synthetized hydrogels in 2.5 ml of distilled water for 24 hr.
    2. Remove the aqueous media where hydrogel is submerged and freeze-dry with liquid N_2.
    3. Laminate the hydrogel sample according to KBr pellet technique.
        1. Add a spatula full of KBr into an agate mortar. Take a small amount of hydrogel sample (about 0.1-2% of the KBr amount, or just enough to cover the tip of spatula) and mix with the KBr powder.
        2. Grind the mixture until the powder is fine and homogenous.
        3. Use the KBr pellet kit to form the IR pellet. Press the powder using a manual laboratory press: for 3 min at pressure capacity equal to 5 tons and then for 3 min at pressure capacity of 10 tons.
        4. Release the pressure to obtain the final pellet as homogeneous and transparent in appearance. Insert the pellet into the IR sample holder and run the spectrum[16].

2. Gelation Studies
    1. Fill 2 ml microcentrifuge tube with 900 µl of PBS and equilibrate to 37 °C.
    2. Add 100 µl of the prepared polymer solution to form the hydrogel and incubate at 37 °C.
    3. Invert the tube and observe if the gel flows at 1, 2, 5, 10 and 20 min. Record the time at which the gel does not flow as the gelation time.

Representative Results

The PAA alkyne derivative is efficiently synthesized from polyacrylic acid and propargylamine, as showed in **Figure 1** where *n* labels the monomers whose carboxyl groups react with the amine. The identity of the product is confirmed by ^{1}H-NMR spectroscopy. **Figure 5** shows the ^{1}H-NMR spectrum of PAA modified with triple bond.

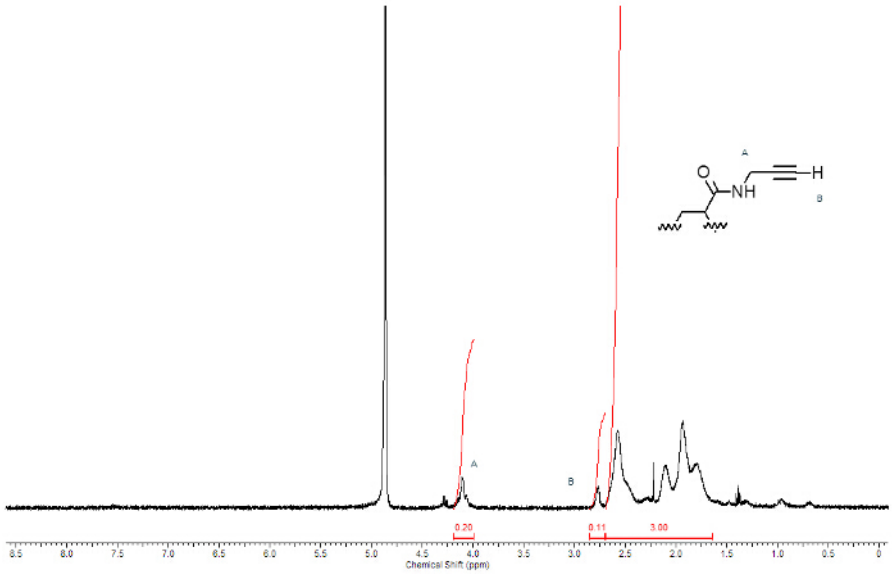

Figure 5: ^{1}H-NMR spectrum of the PAA modified alkyne. The signal related to the alkyne moiety is highlighted. Please click here to view a larger version of this figure.

The signals of the polymer chain can be observed in the range 2.75-1.50 ppm; whereas a peak at 2.80 ppm, representative of alkyne's H, and a peak at 4.20 ppm, related to the 2 H of the -CH_2, characterize the propargyl moiety. This confirms that PAA has been properly modified. The evaluation of the degree of alkyne functionalization has been carried out by integrating the area under the PAA peaks (set to 3.00, according to the number of hydrogens per monomer) and propargyl moiety, as illustrated in **Figure 5**. The degree of functionalization *f* is calculated as:

$$f = \frac{A_{propargyl}}{A_{PAA}} \times 100 = \frac{A_{H_{alkyne}} + A_{CH_2}}{A_{PAA}} \times 100$$

$A_{propargyl}$ represents the integral area of the propargyl residue, the sum of the alkyne's H area (labelled as $A_{H_{alkyne}}$) and the -CH_2 area (indicated as A_{CH_2}), whereas A_{PAA} refers to the integral area of the polymer signals. The degree of functionalization is calculated to be 10% and it is consider satisfactory according to the hydrogel synthesis, where PAA has to react through its residual carboxyl groups to form the 3D network. A quantitative yield is obtained for the modified polymer[16].

In a similar manner, **Figure 6** shows the ^{1}H-NMR spectrum of the product after the CuAAC click reaction between the alkyne modified PAA and RGD-azide. The peak of the formed triazole at 8.15 ppm confirms that the reaction occurs in a quantitative yield and RGD is strongly linked to the PAA chains. **Figure 6** illustrates all of the characteristic signals of the PAA chain and the RGD.

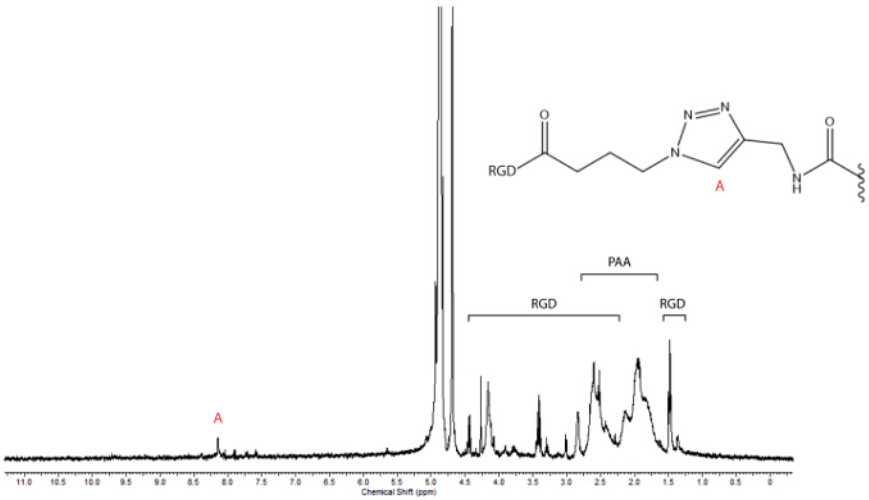

Figure 6: ^{1}H-NMR spectrum of the RGD linked to PAA. The signal of triazole is indicated (labelled as "A"). RGD polymer functionalization via CuAAC click reaction is performed. Please click here to view a larger version of this figure.

RGD-functionalized hydrogels are prepared through chemical cross-linking of the four polymers (PAA, carbomer, agarose and PEG) by microwave-assisted free radical polymerization. Heating to 80 °C leads to a higher macromer mobility, and thus enhances the short-range interconnections among the carboxyl and hydroxyl groups of the polymers. The esterification reaction takes place between these functional groups and produces local networks called "microgels".

As the polycondensation proceeds, the system viscosity increases continuously, while the probability of interaction between macromer reactive sites decreases. Nevertheless, the closer functional groups still interact efficiently due to a slower mobility. The resulting physicochemical condition is characterized by a "welding" between microgel surfaces that produces the final 3D macrostructure of the hydrogel. The esterification, hydrogen bonding and carboxylation bring the polymer chains statistically closer, thus creating a stable heterogeneous structure. The resulting system exhibits sol/gel behavior and it transitions to a gel state within 5 min. This time interval is reported as gelation time.

The chemical nature of the RGD-functionalized hydrogels is studied using FT-IR analysis. **Figure 7** shows the comparison among FT-IR spectra of the RGD-azide compound (green line), the hydrogel synthesized without RGD functionalization (black line), and the hydrogel with peptide modification (blue line). The hydrogel spectra are both characterized by a broad signal in the 3,600-3,200 cm^{-1} range, representative of the stretching vibration of residual O-H bonds and by a peak around 2,940 cm^{-1} of the C-H stretch. The validation that esterification occurs among the carboxyl and hydroxyl polymer groups is given by the peaks around 1,600 cm^{-1} and 1,400 cm^{-1}, corresponding, respectively, to the symmetric and asymmetric stretching of CO_2 moiety. These peaks are more visible in the spectrum of the non-functionalized hydrogel, whereas in the RGD-hydrogel spectrum they are partially covered by the signals indicated as amide bands I and II.

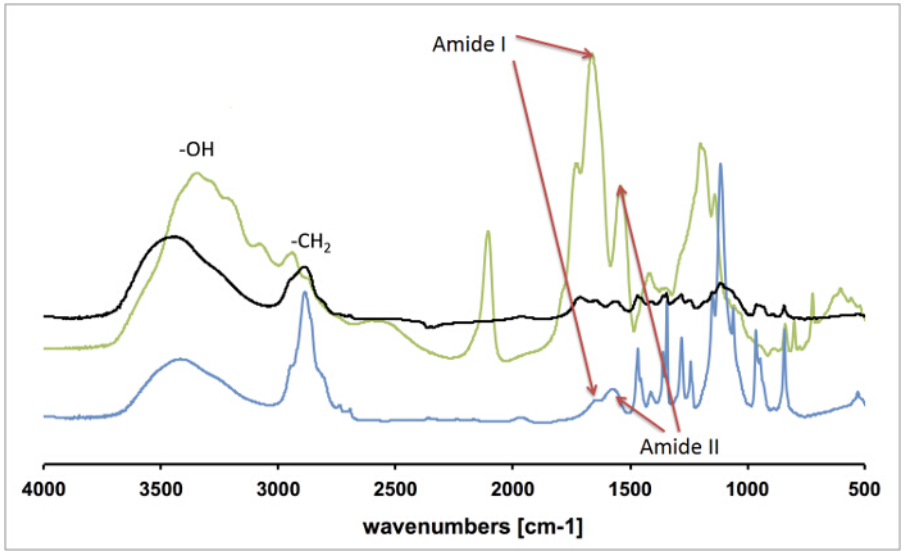

Figure 7: Comparison of FT-IR spectra. FT-IR spectra of RGD (green line), hydrogel without RGD functionalization (black line) and RGD functionalized hydrogel (blue line). The signal related to the amide RGD is indicated. Please click here to view a larger version of this figure.

The stretching of C=O, labelled as amide band I ("Amide I" in **Figure 7**), presents a peak at 1,650 cm^{-1} in the tripeptide spectrum and it is shifted to about 1,670 cm^{-1} in the RGD-hydrogel sample. The bending of N-H, related to amide band II ("Amide II" in **Figure 7**), can be recorded with the signal around 1,550 cm^{-1} in the RGD spectrum and it is also recognizable in the hydrogel sample, at around 1,600 cm^{-1}. Because there are no

amide components in the standard hydrogel formulation, the presence of peaks of an amidic nature suggests that the PAA is really functionalized with the RGD and it is able to form a hydrogel with peptide sites within the polymeric network.

The hydrogel FT-IR spectrum also shows the peaks related to the stretching vibration of C-O-C of glycosidic bond (900-1,000 cm^{-1} range) between the monosaccharide units of the agarose and the ester groups.

To obtain insight into the 3D structure and physical and mechanical properties of these hydrogels, SEM analysis, gelation, swelling kinetics and rheological studies are performed, as discussed in previous works[13,20]. SEM results (**Figure 8**) show that hydrogels are characterized by a complex microscopic structure with some bigger pores containing small pores and some fibrillar networks on the pore walls. In addition, most of the pores are interconnected. The entangled structure is similar to the 3D network of hydrogels prepared in the same manner but without RGD functionalization. This demonstrates that the RGD does not alter the polymer network. Using the inverted test tube test, the hydrogel sample solidifies within 5 min, as observed in the hydrogel sample without RGD functionalization[21]. This short gelation time underlines its suitability for biomedical applications.

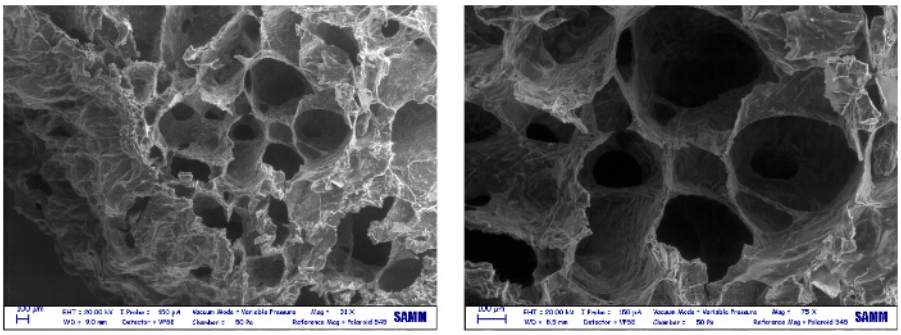

Figure 8: SEM analysis. SEM images show the morphology of a RGD-functionalized hydrogel sample (**A**) and a hydrogel without functionalization (**B**). Please click here to view a larger version of this figure.

The swelling equilibrium ratio indicates the ability to absorb and retain a large amount of water and it is one of the leading features of hydrogel systems[20,22]. The analyzed samples exhibit fast swelling kinetics and they reach swelling equilibrium within the first hour. Their swelling equilibrium value Q is reported in our previous work[16] and it is similar to the value obtained by analysis of hydrogels without RGD, confirming that the tripeptide is integrated with the polymeric network and does not create a high hindrance to the gelation process.

With the rheological studies, the gel storage modulus (G´) is found to be approximately one order of magnitude higher than the loss modulus (G´´), indicating an elastic rather than viscous material[23] and both are essentially independent from frequency. Similar values of G´ and G´´ are recorded with the gel sample without a peptide modification[16]. This demonstrates that the presence of RGD within the polymeric network does not affect the rheological properties of the material, maintaining the peculiar features competing to injectable system for biomedical application.

Discussion

The PAA post-polymerization modification with alkyne moieties and the RGD functionalization with the azide group guarantee the formation of a stable bond between the polymer and the peptide. Indeed, triazole serves as a rigid linking unit among the carbon atoms, attached to the 1,4 positions of the 1,2,3-triazole ring and it cannot be cleaved hydrolytically or otherwise. In addition, triazole is extremely difficult to oxidize and reduce, unlike other cyclic structures such as benzenoids and related aromatic heterocycles[24]. In this way, the chemical lability of PAA-RGD product is almost absent under the experimental conditions of hydrogel synthesis. The peptide is grafted to the 3D polymeric network due to the nature of its chemical bond and it is not physically entrapped (therefore, it could leave the system).

The alkyne PAA is prepared using propargylamine in an aqueous solution, in the presence of EDC and HOBt, to obtain the amine derivative. Experimental results indicate that the percentage of triple bond linking to the PAA is proportional to the amount of amine used. Referring to the NMR analysis, the polymer functionalization degree is between 90% and 100% when the molar ratio between the reagents PAA and propargylamine is 1:1 and is about 50% when the ratio is 1:0.5. Due to the fact that the formation of the gel network involves an esterification reaction, it is necessary to maintain some PAA carboxyl groups capable of reacting with hydroxyl groups of PEG and agarose. The modification of half PAA carboxyl groups is adequate for this, but hydrogels with improved physicochemical properties are obtained when the polymer functionalization degree is equal to 10% (molecular ratio PAA:propargylamine 1:0.1)[25]. The purification of the alkyne PAA is performed by dialysis, using a dialysis membrane with molecular weight cut-off (MWCO) of 3.5 kDa, allowing the removal of by-products and unreacted species. The product is finally isolated using lyophilization.

RGD is characterized by the amino terminal group -NH_2, which can be used as a coupling point to link the *spacer*-N_3. The linkage between these compounds is obtained via an amide bond that is very stable and not too difficult to form[16]. In order to proceed in this way, 4-azidobutanoyl chloride is prepared as the linker of the *spacer*-N_3 system.

The alkyne moieties react with the RGD-azide derivative, through a click reaction, using copper iodide as a catalyst and sodium ascorbate as an antioxidant agent due to the possible oxidation of copper ($Cu^+ \rightarrow Cu^{2+}$) in experimental atmospheric conditions. After screening different solvents and temperatures, the best results are performed by using a tetrahydrofuran/water mixture at 60 °C[16]. According to the biological application, the post-synthesis retention of copper poses potential toxicity risks, especially for applications where the cells must remain viable. As a solution, dialysis is used to remove the copper catalyst and other by-products, obtaining a purified PAA-RGD compound suitable for hydrogel synthesis without any possible side effects. Click chemistry ensures a quantitative yield of the polymeric product grafting RGD.

Due to the same reactivity of each PAA carboxyl group, the polymer functionalization is statistically evaluated. The exact number of modified carboxyl groups per PAA chain cannot be established and the reported functionalization degree is referred as the total number of carboxyl groups that have reacted with propargylamine. This estimation affects the subsequent assessment of the RGD functionalization and its effective amount loaded into each hydrogel. In addition, GPC analysis is not performed because in this work, the polymer is used and the protocol does not begin from monomers. The percentage of RGD functionalization is commonly verified with ^{1}H-NMR and it is not possible to obtain reliable data from HPLC analysis. However, considering that each step of this work is replicated many times and the same experimental values and results are recorded, the illustrated results are reliable.

Another limitation is the use of copper as the catalyst as previously reported. The possible interaction of the copper with the PAA carboxyl groups during the click reaction and its subsequent presence as an entrapped molecule in the hydrogel network would reduce the biocompatibility of the system and would generate toxic effects in cell cultures. The three-day dialysis minimizes this potential limitation of the technique.

The functionalized PAA is used as a subunit in hydrogel formation, with carbomer, PEG and agarose. Carbomer and agarose are chosen because of their well-known biocompatibility in therapeutic approaches and tissue engineering. Carbomer also has anti-inflammatory properties while agarose is a suitable polymer for tissue repair strategies and provides a good environment for cell adhesion, spreading, proliferation, and protein drugs. Carbomer is a cross-linked poly-acrylic acid containing carboxyl groups (65%) that make it an ionizable molecule. The molecular weight is 10^6 Da. Its use is fundamental for gel synthesis due to its high number of reactive carboxyl groups. However, at the same time, it is very difficult to estimate a quantitative yield of any possible chemical functionalization because of its complex structure. For this reason, PAA is used due to its lower molecular weight (10^5 Da) and its linear structure. The combination of carbomer and PAA allows the design of gel networks with a RGD moiety that, otherwise, would not be viable. PEG is also introduced into the gel network due to its high biocompatibility, its ability to increase stem cell adhesion, and its high elasticity (being a small polymer) that can create elastic junctions between the carbomer and the agarose to form local interconnections through their own hydroxyl groups[26]. It also has a physical effect by increasing system viscosity and thus enhancing tight macromer cross-linking.

The use of the four polymers leads to a 3D network that is highly biocompatible, injectable and capable of maintaining its biochemical functionality with improved cell adhesion due to the presence of RGD. The latter is decisive in generating a suitable cell microenvironment within the hydrogel that otherwise would not give a satisfactory result[27].

The hydrogel network can be consider as a "carbon skeleton" of macromolecules forming a stable structure comprised mostly of C-C bonds, where the cross-linking points are C-O bonds formed by esterification between carboxyl and hydroxyl groups. The microwave irradiation is a key point to ensure the agarose solubility in the aqueous reagent system and to avoid any further purification in the synthesis of sterile hydrogels via the sol/gel transition, because any initiators or monomers can be used and by-products are not formed. The microwave-assisted reaction also guarantees a high efficiency, and the functionalization degree of polymer chains with bioactive compounds or drugs generally does not affect the morphology, the structure, and the physical properties of the networks. These hydrogels are able to form *in situ* due to their ability to easily conform to any shape and be injected using minimally invasive surgical procedures. This is a significant advantage compared to the use of rigid and preformed scaffolds.

The synthesized networks can be used as an enhanced cell survival biomaterial, thanks to its high biocompatibility and the ability to create a hospitable cell microenvironment[27]. The improvement of this work is the ability to design a three-dimensional structure where the RGD is chemically linked through a stable and strong bond that is not cleavable in physiological conditions, thus avoiding RGD diffusion through the hydrogel pores to maintain its therapeutic effect at a high level for a long and sustained period of time. Alternative synthesis of chemical linkers, such as the ester or the hydrazone bonds, do not allow the same effect towards cells because they are cleavable, or pH/temperature sensitive under the cell microenvironment[21,28,29]. In addition, the tripeptide release is mostly driven by a pure diffusion mechanism that is very quick due to its small steric hindrance. The alternatives to modifications of the physical and mechanical parameters of the hydrogel network, for example reducing pores size, cannot be enough to maintain an adequate concentration of RGD within the matrix.

A possible additional functionalization is the use of fibronectin, which can optimize the hydrogel compatibility with the extracellular matrix in order to make viable living cells. One of the most important aspects is maintaining cell viability within hydrogel networks for days. In this way, cells can leave the matrix able to proliferate and differentiate *in vivo*. The first experimental results are published[27]. This highlights the comparison between RGD-functionalized hydrogels and non-functionalized hydrogels showing promising results. In particular, the RGD in the gel network improves stem cell survival and guarantees the immunomodulation of the pro-inflammatory and pro-regenerative effect at the lesion site *in vivo*. The presence of RGD linked to the hydrogel is a crucial point to achieve suitable therapeutic results. The RGD maintains its availability for cell interactions and it does not escape rapidly through hydrogel pores, maintaining its bioactive impact.

Disclosures

The authors state no conflict of interest and they have not received any payment in preparation of this manuscript.

Acknowledgements

Authors would like to thank Prof. Maurizio Masi for fruitful discussion and Miss Chiara Allegretti for language editing. Authors' research is supported by Bando Giovani Ricercatori 2010 (Ministero della Salute GR-2010- 2312573).

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