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**SYNTHESIS OF TEMPO GRAFTED HYDROGELS  
AS POTENTIAL AGENTS FOR MRI**

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*“Perché la ruota giri, perché la vita viva,  
ci vogliono le impurezze.  
Ci vuole il dissenso, il diverso,  
il grano di sale e di senape.”*

Primo Levi

# Sommario

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Da più di un decennio gli idrogel vengono studiati come materiali per il drug delivery e per l'ingegneria tissutale, grazie alla loro struttura flessibile e l'elevata biocompatibilità. Non è molta, tuttavia, la letteratura scientifica relativa all'uso degli idrogel come mezzi di contrasto, anche se in questo campo d'applicazione si stanno attualmente sviluppando interessanti prospettive.

Gli idrogel, per esempio, potrebbero essere usati come carrier di un farmaco e simultaneamente permettere il monitoraggio del suo rilascio attraverso la risonanza magnetica per un periodo di tempo biologicamente significativo.

Lo scopo di questa tesi è la sintesi di nuovi idrogel come agenti di contrasto per la risonanza magnetica. A questo fine, gli idrogel sono stati funzionalizzati con il TEMPOL, che è uno spin label paramagnetico e quindi può essere monitorato attraverso la risonanza magnetica.

Il TEMPOL costituisce un materiale particolarmente interessante perché rappresenta un'alternativa al Gadolinio, attualmente usato come agente di contrasto nonostante i rischi legati alla sua tossicità. Rispetto al Gadolinio, infatti, il TEMPOL presenta molti vantaggi: tempi di rilassamento relativamente elevati, una prolungata emivita, eccellente biocompatibilità e sicura biodegradabilità.

I polimeri funzionalizzati, scelti per la sintesi degli idrogel, sono basati su PAA e PEG. In tutti i casi sono stati effettuati differenti approcci per connettere il polimero con il TEMPOL, sfruttando sia reazioni di click chemistry che metodi di sintesi più classici.

Abbiamo infine anche indagato, in modo preliminare, la possibilità di sintetizzare degli idrogel funzionalizzati con  $\beta$ -ciclodestrine-TEMPO, in collaborazione del gruppo di ricerca del Prof. Punta.

In ultimo, l'efficacia dei nostri sistemi è stata poi testata mediante prove sperimentali di carattere biologico grazie alla collaborazione con l'Istituto di Ricerca Farmacologica Mario Negri di Milano.

# Abstract

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For more than a decade, hydrogels have been extensively studied and used as drug delivery media and tissue repairing materials, benefitting from their softness and excellent biocompatibility.

Literature on directly labeling hydrogels with a contrast agent is not so common, even if this field shows many prospective applications. For instance, hydrogels can be employed for carrying drugs and their release *in vivo* could simultaneously be monitored with the help of MRI over a prolonged period.

The aim of this thesis is the synthesis of new hydrogels as contrast agents media for MRI. For this purpose, hydrogels are labelled by TEMPOL, which is a paramagnetic spin label and, hence, can be detected by MRI.

TEMPOL is particularly attractive because it is an alternative to Gadolinium, the current most common probe, with the drawback of being highly toxic for human body. Compared to Gadolinium, TEMPOL shows many advantages, such as comparatively high relaxation time, prolonged blood half-life, excellent biocompatibility and safe biodegradation properties.

In our case, the required functionalized polymers for hydrogels syntheses are based on PAA and PEG. Two different approaches were applied to functionalize the polymers with TEMPOL: on the one hand we took advantage of click chemistry, on the other hand we exploited alternative and more classical organic syntheses. In addition, we have exploited the possibility to form  $\beta$ -cyclodextrins-TEMPO hydrogels, synthesized in collaboration with Prof. Punta's group.

Finally, the effectiveness of our materials has been evaluated through biological assays at Mario Negri Institute for Pharmacological Research.

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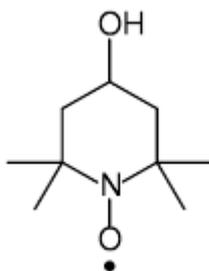
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# Introduction

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## *4-HYDROXY-TEMPO*

An organic molecule with an unpaired electron and able to bind to another molecule is known as a spin label. Spin labels are normally used as tools for probing proteins using Electron Paramagnetic Resonance (EPR). Among the spin labels, the most common are the so called nitroxides, even though IUPAC rules define compounds with the structure  $R_2NO\bullet$  as aminoxyl radicals [1]. The most extensively studied nitroxide is TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl), as shown in Fig. 1.



*Fig. 1: Chemical structure of 4-hydroxy-TEMPO or TEMPOL, formally 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl*

In 1965 Mc Connell demonstrated that nitroxides are “free radicals” and paramagnetic “spin labels” [2]. He developed the technique of spin labelling, whereby electron and nuclear magnetic spectra can be used to study the structure and the kinetics of proteins and membranes. His investigations laid a firm foundation for the analysis of the paramagnetic resonance spectra of organic free radicals in organic structures, further showing that nitroxides could be linked stably and covalently to proteins and other agents as biomarkers for molecules of interest [3].

Nitroxides in fact are stable radicals without significant plasma proteins binding. The presence of a single unpaired electron on the radical yields insights of its pharmacokinetics, because this species is detected by MRI (Magnetic Resonance Imaging) or by a characteristic spectrum on EPR. These signals are lost after the bioreduction of the nitroxide to the diamagnetic hydroxylamine. The rate of TEMPOL

reduction in an organ is related to ROS production [2]. ROS (Reactive Oxygen Species) are oxidant compounds produced in the glucose-oxidation process. The oxidative damage within the living body is governed by the balance between the production of ROS and the production of antioxidant protective systems (e.g. ascorbic acid, super oxide dismutase [SOD] ). [4]

### OXIDATIVE STRESS

Oxidative stress is an uncontrolled production of ROS, able to compromise the balance between oxidant and anti-oxidant systems in living beings. ROS are formed by the sequential reduction of oxygen, generating different oxidant species through a cascade of electrons additions. Examples of ROS are, for instance, molecules such as hydroxyl, alkoxy, peroxy and superoxide radicals (Fig. 2).

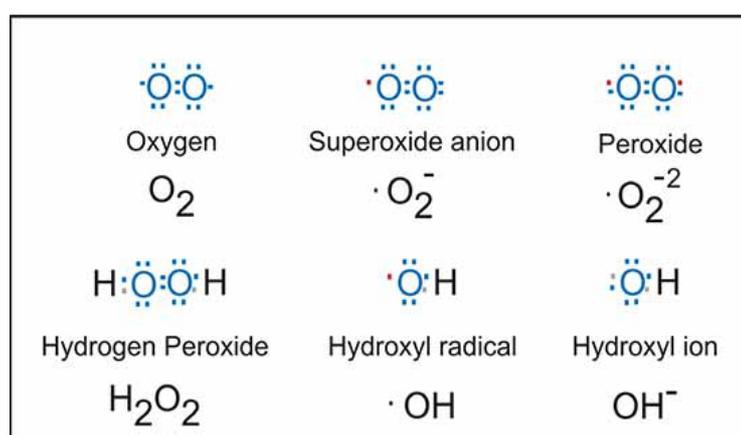


Fig. 2: Electronic structures of common reactive oxygen species

ROS are short-lived diffusible entities, highly unstable and active towards chemical reactions with other molecules. An increase of ROS within the cells induces damages mainly on proteins, DNA, RNA molecules and causes an oxidative stress in the cells.

An uncontrolled oxidative stress is involved in the origin and development of many pathologies, including chronic inflammation, cancer, renal failure and the deposition of arterial plaques in atherosclerosis.

Antioxidants can scavenge ROS or inhibit ROS production and they are often reducing agents such as thiols, ascorbic acid or polyphenols. However, most of them, such as vitamins, non- flavonoid and flavonoid compounds, appear not to be suitable drug

candidates, because they show poor solubility and inability to cross membrane barriers [5]. Nitroxide compounds are becoming interesting for protection against oxidative damages due to their scavenger activity, even though they have been mainly used as tools for spectroscopic studies, because of their stability and their paramagnetic nature *in vivo* and *in vitro*.

Under *in vivo* conditions, however, these radicals pose severe problems, such as non-specific distribution in normal tissues, high rate of renal clearance and rapid first pass metabolism, with their reduction and subsequently inactivation to the corresponding hydroxylamine [4].

### BIOCHEMISTRY OF NITROXIDES

Cyclic aminoxyl radicals with tetra-alkyl substituents flanking the NO• functionality, such as TEMPO and its derivatives (Fig. 3), are most often used as probes for the redox status *in vivo* [5].

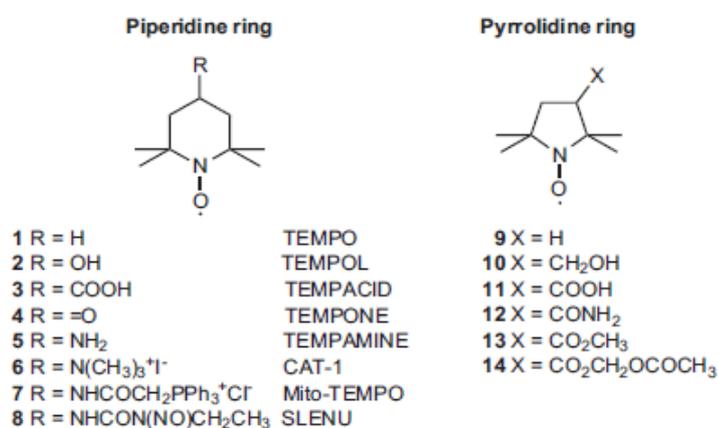


Fig. 3: Aminoxyl radical used as probes of the redox status [6]

These radicals are called stable free radicals but they do participate to the redox status of the cell *in vivo* (Fig. 4).

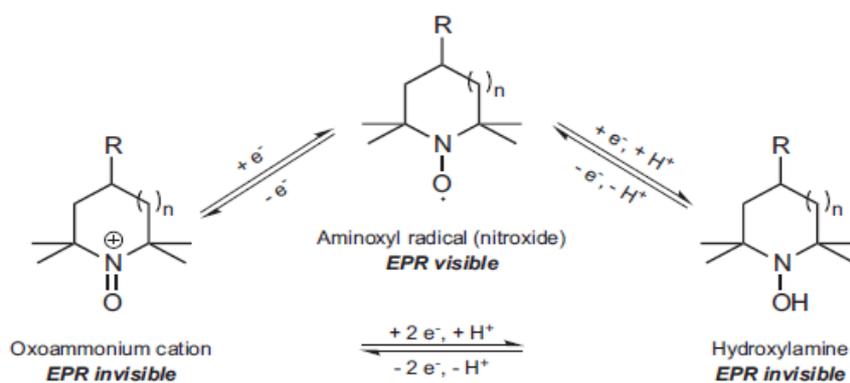


Fig. 4: Redox species associated with aminoxyl radicals [6]

In biological systems, the reduction of nitroxides radical to hydroxylamine occurs within the cells by means of ascorbic acid, and it is largely dependent on the ring size.

Piperidine ring based nitroxides are reduced faster than the pyrrolidine ring based ones; moreover anionic compounds (3) are more stable than neutral compounds (2,4) which are more stable than the cationic ones (5,6). An increased concentration of oxygen within the cells can lower the reduction rate but this regards both the cell type and the lipophilicity of the nitroxide [6].

## CONTRAST AGENTS

The contrast agents are substances used to enhance the contrast of structures or fluids within the body in medical imaging. In MRI (Magnetic Resonance Imaging) the parameters that affect the signal are related not only to the proton density, like radiography, but especially to the proton relaxation. This has made possible to develop products that can significantly increase the contrast between normal low dense tissues and pathological ones even at very low concentration, because MRI contrast media affect the relaxation times T1 and T2 of the protons arranged in their adjacency.

The relaxivity quantifies the change induced in T1 or T2 as a function of concentration. In a constant magnetic field, the relaxivity is defined as follows:

$$1/T_{1,2} = 1/T_{1,2}^0 + r_{1,2} [C]$$

where  $T_{1,2}$  indicates the resulting T1 or T2,  $T_{1,2}^0$  indicates the T1 or T2 of the original tissue,  $r$  is the relaxivity and  $[C]$  is the concentration of the contrast medium in the tissue [5].

Gadolinium-based contrast agents (GBCAs) are the most widely used paramagnetic metal ion-based agents in the clinic.

Although GBCAs are well-tolerated by the majority of patients, some of them, especially if with impaired kidney functionality, have increased risk of developing a serious adverse reaction named nephrogenic systemic fibrosis (NSF) [7].

In addition, Gadolinium is known for being highly toxic for human body, so that it is synthesized as a chelate with an agent that minimizes the risk of the release of the metal when used for MRI. Although Gadolinium contrast agents have good track record in tumor diagnostics, they are neither specifically targeting to malignant tissue nor they are actually cell permeable [8].

The World Health Organization issued a restriction in November 2009 stating that: “Gadolinium-containing contrast agents are contraindicated in patients with severe kidney problems, in patients who are scheduled for or have received a liver transplant and in newborn babies up to four weeks of age” [9].

Therefore, ORCAs (Organic Radical Contrast Agents) would provide an alternative to GBCAs. To date, the critical obstacle in the development of ORCAs for MRI is the

design and synthesis of paramagnetic compounds of moderate molecular size that have sufficiently long lifetimes *in vivo* and high water solubility.

Paramagnetic nitroxyl radicals have been proposed as redox-sensitive MR contrast agents. Compared to Gadolinium, nitroxides have also shown advantages, such as comparatively high relaxation time, prolonged blood half-life, excellent biocompatibility and safe biodegradation properties [10].

The decay rate of the signal level, that is, the reduction rate of the nitroxyl radical, is faster in a tumor tissue than in a normal one. This difference in the decay rate of the nitroxyl radical is ascribed to the difference of oxygen levels between the tumor and the normal tissues. However, the relatively small T1 relaxivity of nitroxyl contrast agents makes it difficult to detect T1-weighted MR signal enhancement at low concentration levels. A higher magnetic field, such as 7 T, may increase the signal-to-noise ratio (SNR), while most clinical machines work in lower magnetic fields, such as 3 and 1.5 T or less [5].

#### *IMAGING OF FREE RADICALS*

Imaging techniques performed by adding external paramagnetic agents were stimulated by the discovery that the rate of reduction of nitroxides in cells and tissues is highly dependent on the concentration of oxygen [5]. An ideal technique should be able to directly and non-invasively measure ROS *in vivo*; for instance fluorescence and luminescence are widely used for studying cells and tissues *in vitro*, but they are hardly applicable for *in vivo* studies due to the low penetration depth of used light in tissues [10]. The most direct technique available for the detection of reactive free radicals *in vivo* is ESR (Electron Spin Resonance), also called EPR (Electron Paramagnetic Resonance). ESR has been used to evaluate the generation of free radicals and the redox status in a variety of tissues and organs.

As reported by G. He et al (2002) [11], oxygenation within the gastrointestinal tract of living mice was mapped through this technique. EPR was performed in mice by intravenous administration of a nitroxide radical redox probe. Immediately after the administration, the radical is detected in most of the tissues in the body but it is largely absent from the head. These nitroxide radicals are, in this case, mainly present in the chest and abdominal regions (Fig. 5).

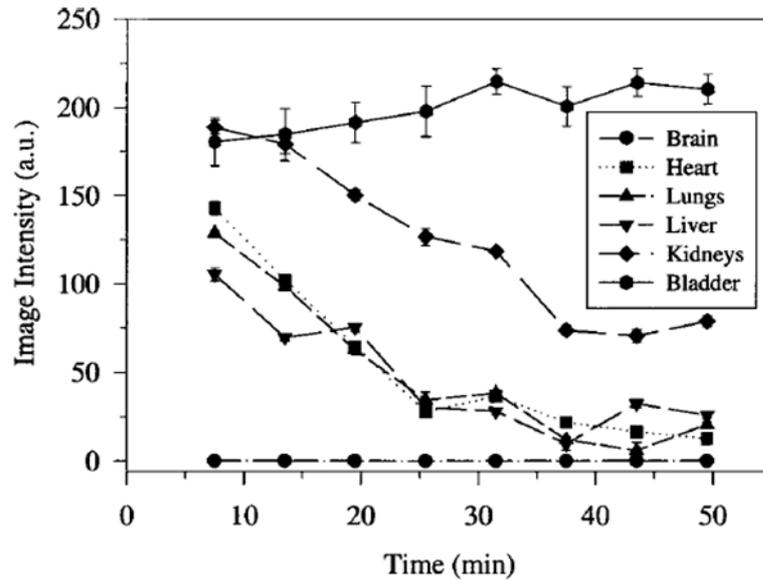


Fig. 5: EPR spectroscopy and EPRI measurement of the time dependence of the distribution and clearance of nitroxide radicals within the whole body and organs of the mouse [11].

The problem with EPR experiments on small animals is the non-resonant absorption of the electromagnetic radiation by the dielectric liquid water in biological systems. This effectively limits the sample size to a thickness of a mouse tail; as a solution increasing the penetration depth of microwaves is not possible because that would produce unacceptable heating of the subject. Hence animals can be studied only by reducing the operating frequency and corresponding magnetic field, but this results in reduced sensitivity [6].

The difference between *in vivo* MRI and EPRI (Electron Paramagnetic Resonance Imaging) is the species they detect.

Endogenous paramagnetic species are present in too low concentrations to be detected directly by EPR *in vivo*. This means the only paramagnetic species that can be detected *in vivo* are the ones introduced by the experimenter.

MRI sees water protons which are abundant, enabling excellent images. Nitroxides are seen indirectly through their effect on the relaxation of water protons.

EPR does not provide images of anatomy; it just shows the distribution of injected nitroxide within the body and does not have very good spatial resolution. Conversely, MRI has excellent spatial resolution and provides detailed anatomical information, but it gives little information on the paramagnetic species involved.

The best way to fuse EPR and MRI into a single machine is to use OMRI (Overhauser Magnetic Resonance Imaging) [6]. OMRI is a double resonance technique that creates images of free radical distribution in small animals by enhancing the water signal intensity via the Overhauser effect. The significant contrast to noise ratio is obtained by this technique at very low magnetic fields (10 mT) which makes OMRI advantageous for physiological investigations [12].

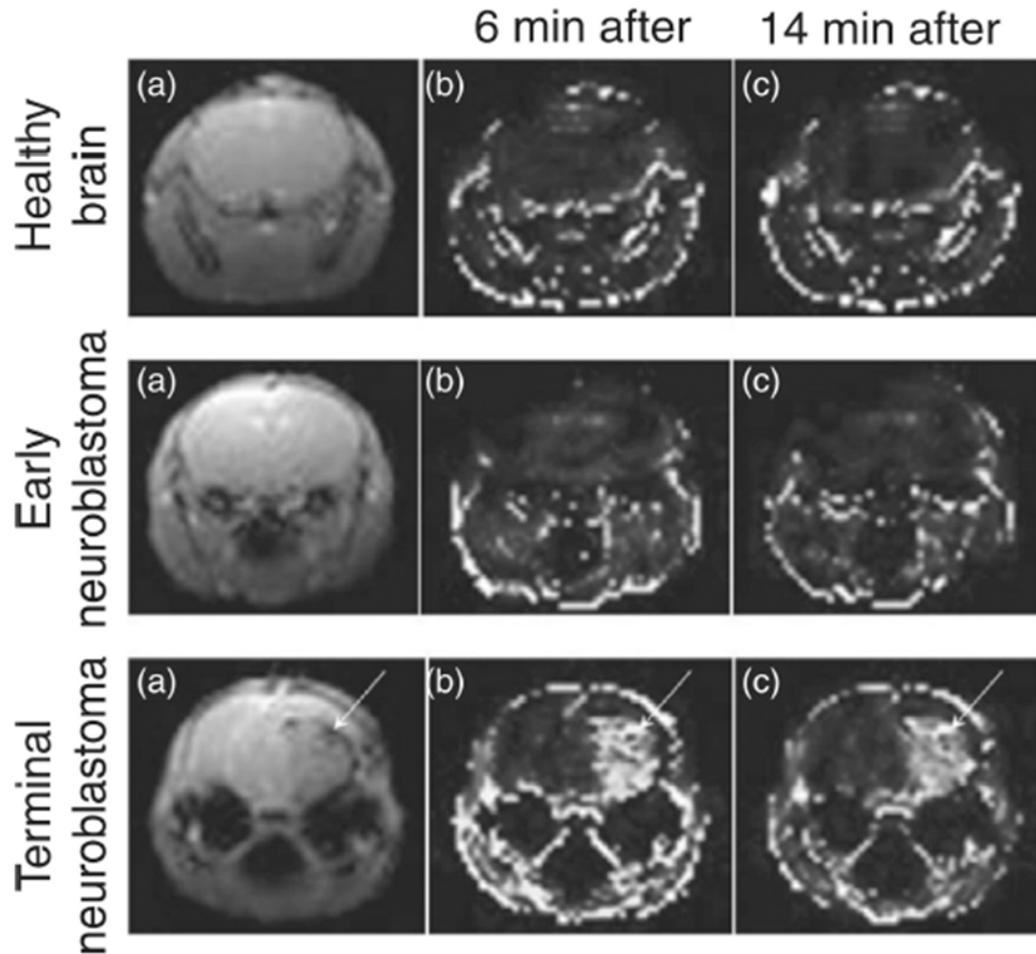
### *SELECTED EXAMPLE*

A lot of pathological conditions have been studied using reduction of nitroxides. In particular, they are useful to obtain a good insight into the redox status of tumors. Cancer cells and tissues have different redox abilities with respect to normal tissues. Given that the efficiency of radiotherapy depends on oxygenation of tumors, the reduction of different nitroxides has been studied by *in vivo* MRI on animal models.

Some general considerations can be made:

- reduction of all cell-permeable nitroxides is faster in a tumor muscle than in a healthy one in all tumor types;
- different reduction rates are associated to different types of tumors, so different reduction rates are the result of different intracellular bioreductions.

Studies performed on brain tumors by monitoring the reduction of SLENU (a TEMPO-labelled analogue), showed that the half-life of nitroxides in early stages of tumors is comparable to ones in healthy mice, but much shorter than in tumors at terminal stages [6], (Fig. 6).



*Fig. 6: Nitroxide enhanced MRI images of healthy and tumor bearing mice in the early and terminal stage of disease [6]*

Nowadays, lipophilic nitroxyl compounds are being attractive because they are non-metal BBB (blood brain barrier) permeable contrast agents. Although brain imaging is one of the main clinical applications of MR, clinically available BBB-permeable contrast agents are currently absent.

## HYDROGELS

Hydrogels are networks of hydrophilic cross-linked polymers, natural or synthetic, containing a large amount of water. They maintain a distinctive three dimensional structure, characterized by different grade of entanglement of the chains, depending on their formulation. The high water content (typically in the swollen state, where the mass fraction of water in a hydrogel is much higher than the mass fraction of polymer) makes it able to simulate tissue microenvironments in term of elasticity. Moreover their capability to carry drugs, growth factors and cells make hydrogels an ideal class of materials for biomedical applications, such as drug delivery and tissue engineering [13].

### NETWORK STRUCTURE AND FUNDAMENTAL PARAMETERS

The network structure of an idealized hydrogel and its characteristic variables are shown in figure 7. They are: mesh size, cross-linkage density, average molecular weight between two following cross-link points and volume, both in dry and swelling state.

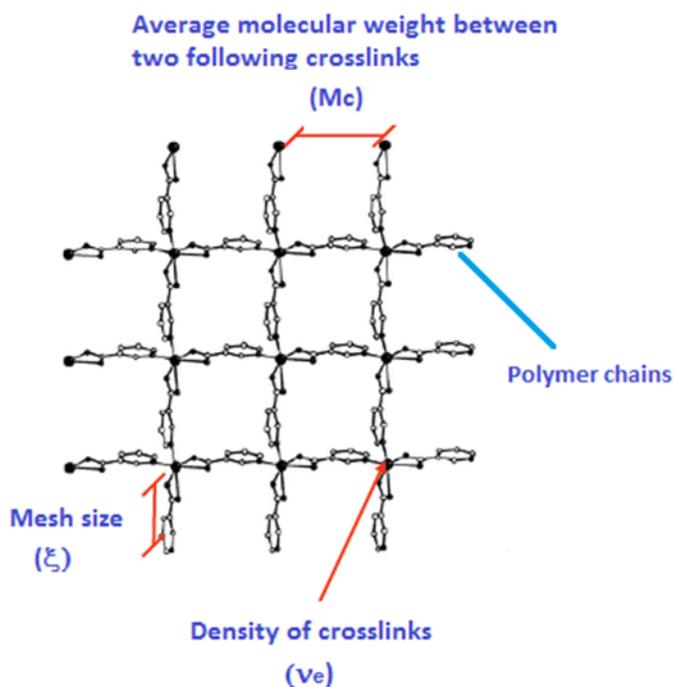


Fig. 7: network structure and main parameters

The most important parameters that define the structures and properties of hydrogels are:

- ◆ the polymer volume fraction in the swollen state, indicated as  $v_s$ . It is defined as the ratio between the polymer volume  $V_p$  and the swollen gel volume  $V_g$ . It is also the reciprocal of volumetric swelling ratio  $Q_v$ :

$$v_s = \frac{V_p}{V_g} = \frac{1}{Q_v}$$

where  $Q_v$  can be defined as:

$$Q_v = 1 + \frac{\rho_p}{\rho_s}(Q_m - 1)$$

$\rho_p$  is the density of the dry polymer and  $\rho_s$  the density of the solvent;  $Q_m$  represents the ratio between the weights of swollen polymer ( $W_{swollen}$ ) and dry polymer ( $W_{dry}$ ):

$$Q_m = \frac{W_{swollen}}{W_{dry}}$$

- ◆ the effective molecular weight of the polymer chain between two following cross-linking points, designated as  $M_c$ . It is related to the degree of gel cross-linking  $X$  and the molecular weight of repeating monomer unit  $M_0$ :

$$M_c = \frac{M_0}{2X}$$

- ◆ the distance between sequential points of crosslink,  $\xi$ , which represent an estimate of space between macromolecular chains accessible for drug or cell diffusion. It can be calculated as:

$$\xi = v_s^{-1/3} * C \left( \frac{M_c}{M_0} \right)^{1/2}$$

where  $C$  is a constant for a given polymer-solvent system.

- ◆ cross-linkage density,  $v_e$ , that is the ratio between polymer density and  $M_c$ :

$$v_e = \frac{\rho_p}{M_c}$$

## DESIGN FEATURES

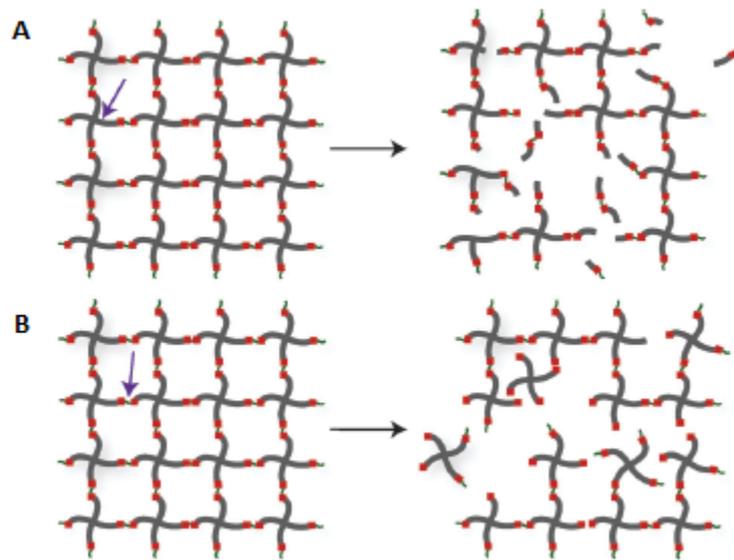
Hydrogels permit control and reproduction of typical properties of cell microenvironment but they should be designed properly.

Key aspects are shown below.

- ◆ **Biocompatibility:** it's the ability of a biomaterial to perform its desired function without any negative local or systemic side effects, such as inflammation or toxicity.
- ◆ **In situ cross-linking:** it's the capacity to create three dimensional scaffold. It is so possible *in vivo* to mold the gel to the shape of the defect site and delivery in a minimally invasive way. It's very important that chemical transformations do not produce compounds (such as free radicals) that could damage cell viability and activity.
- ◆ **Mechanical properties:** they influence drug's compatibility and loading, cell migration, proliferation and differentiation. These properties can be tuned through polymers concentration, stoichiometry of reactive groups and cross-linking density.
- ◆ **Mass transport:** in tissue engineering, continuous exchange of nutrients, proteins, gases (for example, O<sub>2</sub> and CO<sub>2</sub>) and waste products into, out of, or within the hydrogel is vital for survival and proliferation of cells; instead, for controlled delivery of bioactive elements (for example, therapeutics or proteins), restricted free diffusion is essential. Hydrogel matrix permeability is a very important design parameter, given that the mass transport in these materials is controlled primarily by diffusion: increasing or reducing permeability is possible to influence the amounts of cargo released by the system. It is also correlated with the mechanical characteristic and swelling behavior of the network.
- ◆ **Microenvironment:** it is related to the capacity of hydrogel to simulate the dynamic nature of extracellular matrix (ECM) to ensure full cell-compatibility. ECM is a complex environment comprising structural proteins, polysaccharides, growth factors, enzymes and inhibitors essential for cell proliferation and migration. Bidirectional cross talk between the microenvironment and resident

cells is called “dynamic reciprocity”. Nowadays, there are multiple strategies to try to generate matrices capable of this reciprocity and including them in the structure of the hydrogels.

- ◆ **Degradation:** hydrogels can be designed to degrade via ester or enzymatic hydrolysis, photolytic or environmental (pH, temperature, solvent quality) cleavage or a combination of these mechanisms, with different degrees of degradation rates depending on the application. They can be broken along polymer chains or at the crosslink points, as shown in figure 8.



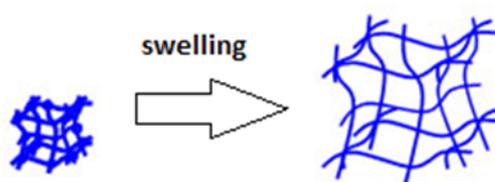
*Fig. 8: two different way of hydrogel degradation.  
A: breaking polymer chain; B: breaking crosslink point.*

Indeed, in tissue engineering uses, degradation provides space to allow cell proliferation, migration, matrix remodeling in order to simulate ECM and infiltration of blood vessels; in drug delivery applications, progressive decay permits spatial-temporal control of the release of cargo molecules. Release kinetics are determined by surface or bulk erosion. In the process of surface erosion, the water is adsorbed on the surface before it diffuses into the bulk of the gel, because water diffusion rate into network is slower than the degraded reaction. Bulk erosion, on the other hand, occurs when the rate of water diffusion is much faster than the hydrolysis reaction. In addition, it is important to generate byproducts biocompatible and without inducing potential side effects, such as cytotoxicity, inflammation or immunological or foreign body responses.

## *SWELLING BEHAVIOUR*

Hydrogels can swell when they are in contact with a thermodynamically compatible solvent.

The molecules of the solvent attack the surface of the hydrogel and they can penetrate into the polymeric network. Regularly, the meshes of network will start expanding, allowing other solvent particles to come up to the core of the scaffold. The result is in figure 9.



*Fig. 9:swelling behavior*

The polar hydrophilic groups are the first to be hydrated upon contact with polar solvent (in general aqueous solutions) which leads to the formation of primary bound water, and then, the hydrophobic groups so exposed are able to interact with the water molecules. This leads to the formation of hydrophobically-bound water, also called secondary bound water. The network will absorb additional water, due to the osmotic driving force of the network chains towards infinite dilution. It is obvious that swelling is not a continuous process: there is an opposition by the covalent or physical cross-links, leading to an elastic retraction force, which balances the stretching of the network and prevents its deformation. Thus, the hydrogel will reach an equilibrium swelling level: in this state, where elasticity and osmotic forces are balanced, there is no further swelling. The additional absorbed water is called “free water” or “bulk water” and assumed to fill the space between the network chains, or the center of macropores, or voids. Depending on the nature and composition of the hydrogel the next step is the disintegration or dissolution, if the network chain or cross-links are degradable.

This context shows the importance of knowing the value of the swelling rate, which is determined by several physicochemical parameters, particularly the extent of porosity and the structure of pores. In this relation, hydrogels may be classified into four classes:

1. non-porous hydrogels;
2. micro-porous hydrogels;
3. macro-porous hydrogels;
4. super-porous hydrogels.

The polymer chains in non-porous gels are densely packed and severely limit solute transport via diffusion through free volumes. The equation developed to characterize this configuration is related to the ratio of the diffusion coefficient of the solute in the scaffold ( $D_{ip}$ ) to the diffusion coefficient of the solute in the pure solvent ( $D_{iw}$ ):

$$\frac{D_{ip}}{D_{iw}} = k_1 \left( \frac{M_c - M_c^*}{M_n - M_c^*} \right) \exp \left( - \frac{k_2 r_s^2}{Q_m - 1} \right)$$

where  $k_1$  and  $k_2$  are parameters based on the polymer structure,  $r_s$  is the solute radius,  $M_c$  is the molecular weight of the polymer chains between cross-links,  $M_n$  is the molecular weight of linear polymer chains prepared using the same conditions in the absence of cross-linking agent and  $M_c^*$  is the critical molecular weight between cross-links below which a solute of size  $r_s$  could not diffuse.  $Q_m$  is the degree of swelling.

The pore size of micro-porous hydrogels is between 100 and 1000 Å. The solute transport is regulated by a combination of molecular diffusion and convection in the water filled pores. The ratio of diffusion coefficient is defined as:

$$\frac{D_{ip}}{D_{iw}} = (1 - \lambda^2)(1 - 2.104 \lambda + 2.09 \lambda^3 - 0.95 \lambda^5)$$

where  $\lambda$  is the ratio between solute diameter and pore size.

Macro-porous hydrogels have large pores (0.1÷1 µm). Since these pores are much larger than the diffusing species, the effective solute diffusion coefficient ( $D_{eff}$ ) can be described by the diffusion coefficient of solute in the water:

$$D_{eff} = D_{iw} \frac{k_p * \varepsilon}{\tau}$$

$k_p$  is the partition coefficient,  $\varepsilon$  is the network porosity and  $\tau$  represent the network tortuosity.

Super-porous gels, instead, are characterized by pores in the range of several hundred micrometers, that can be connected to form a capillary system causing a rapid water uptake into the porous structure. They allow fast swelling, good swelling ratio and mechanical properties, particularly elasticity.

## SENSITIVITY TO ENVIRONMENTAL CONDITIONS

Many polymers exhibit phase transition property when some environmental parameters change, especially temperature and pH.

About temperature, many polymers increase their solubility in water as the temperature increases, while materials with lower critical solution temperature (LCST) decrease their capacity to dissolve with the same trend of temperature. Hydrogels synthesized starting from this last type of polymers shrink as the temperature is over the LCST, due to the hydrophobic interaction among hydrophobic segments of the polymer that become strengthened. An example of these behaviors is represented in figure 10.

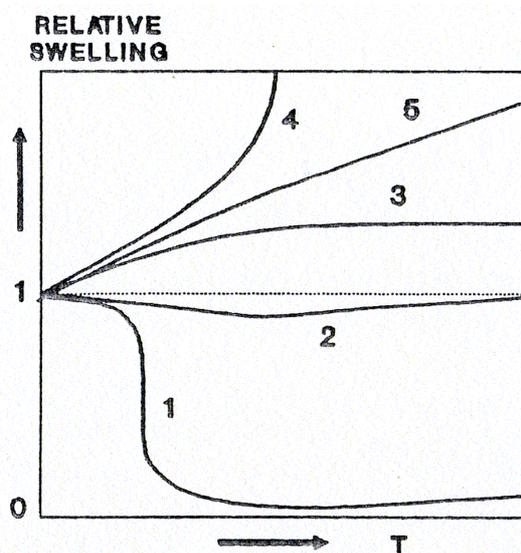


Fig. 10: *poly-isopropyl-N-acrylamide* (trend number 1) and *poly 2-HEMA* (number 2) decrease their swelling at high temperatures, while hydrogels called *HYPAN* (number 3), *HPU* (number 4), and *HYPAN TN* (number 5) have opposite behavior.

If the polymer chains in hydrogels are not covalently cross-linked, temperature-sensitive systems may undergo sol-gel phase transitions, instead of swelling-shrinking switches. The passage to sol happens at high temperature. Polymers that show this type of behavior are block copolymers of polyethylene oxide and polypropylene oxide, commercially available under the name of Pluronics and Tetronics.

In the case of hydrogels made of cross-linked polyelectrolytes, pH has relevant effect in swelling properties. Acidity encourages swelling of basic networks; instead, acid

hydrogels swell more if pH has a value higher than 7 (as illustrated in figure 11): this attitude is a consequence of repulsion between same charges ions. In addition, in amphoteric structures there is a minimal swelling at the isoelectric point. Generally on neutral hydrogels there are any significant pH effect. A summary graphic representation is shown in figure 12.

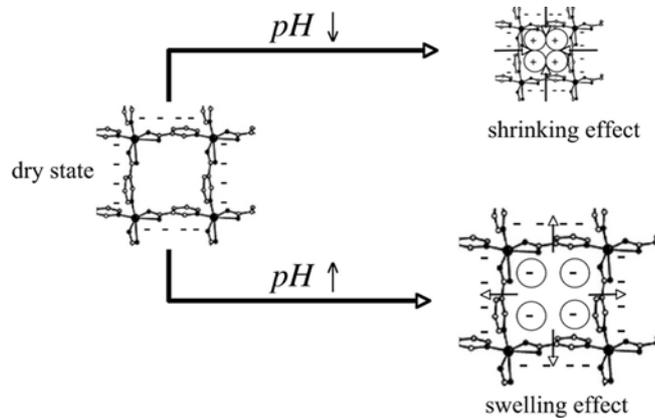


Fig. 11: pH values higher than 7 encourage swelling

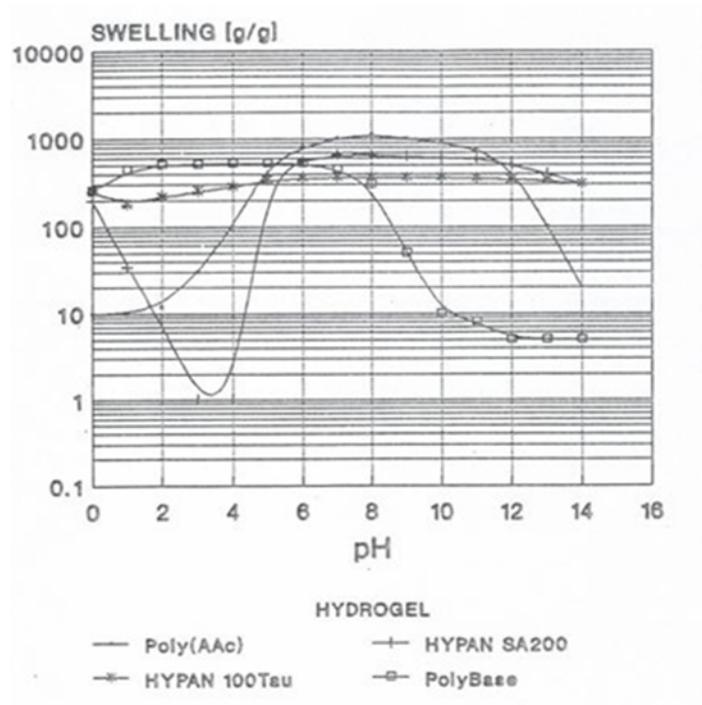


Fig. 12: pH effects on different hydrogel

Hydrogels can be sensitive to other physicochemical variables yet. Those that are glucose-sensitive have an important role in the development of self-regulated insulin delivery system; electro-sensitive gels change in shape, swell or shrink in the presence of an applied electric field and can be used in controlled drug delivery; light-sensitive systems swell in response to UV irradiation or can be activated by visible light; pressure-sensitive structures collapse at low pressure and expand at higher value of it; specific ions or antigens are able to influence crosslink stability and the swelling of hydrogels.

### *CLASSIFICATION*

It is possible to classify hydrogels using different criteria.

#### ◆ *Classification based on source*

- 1) Natural hydrogels, if the polymers are natural;
- 2) Synthetic hydrogels, with polymers characterized by synthetic origin;
- 3) Hybrid hydrogels, if they are composed by both natural and synthetic polymers.

#### ◆ *Classification based on polymeric composition*

- 1) Homopolymeric hydrogels, produced by a single species of monomer which build network;
- 2) Copolymeric hydrogels, that are comprised of two or more different monomer species with at least one hydrophilic component, arranged in a random, block or alternating configuration along the chain;
- 3) Multipolymer Interpenetrating polymeric hydrogel (IPN), consisting in two independent cross-linked polymers, intermeshing to form a network.

#### ◆ *Classification according to structural configuration*

- 1) Amorphous hydrogels;

- 2) Semicrystalline hydrogels;
- 3) Crystalline hydrogels.

◆ *Classification based on type of crosslinking*

- 1) Chemically crosslinked networks, with permanent junctions;
- 2) Physical networks, with ionic or hydrophobic interactions, hydrogen bonds or Van der Waals forces.

◆ *Classification based on physical appearance*

- 1) Matrix system;
- 2) Film;
- 3) Microsphere.

◆ *Classification according to network electrical charge*

- 1) Non-ionic hydrogels (neutral);
- 2) Ionic hydrogels;
- 3) Amphoteric electrolyte containing both acidic and basic groups;
- 4) Zwitterionic hydrogels, containing both anionic and cationic groups in each structural repeating unit.

## *PRODUCTION'S TECHNIQUE*

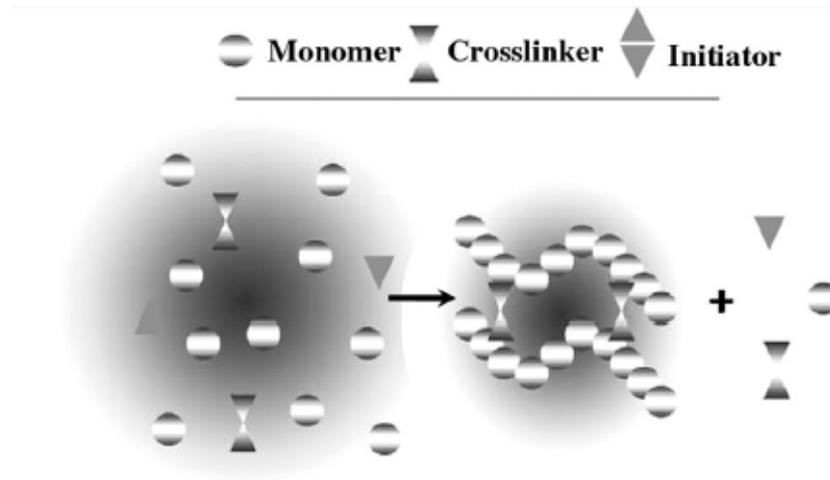
In general, hydrogels can be prepared from either synthetic or natural materials.

Water-soluble linear polymers of both origins can be cross-linked to form hydrogels in different ways:

- ◆ linking polymer chains via chemical reaction;
- ◆ using ionizing radiation to generate main-chain free radicals which are able to recombine as crosslink junctions;
- ◆ polymerizing monomer on the backbone of a preformed polymer, activated by the action of chemical reagent or high energy radiation treatment;
- ◆ through physical interaction such as entanglements, electrostatics and crystallite formation, with or without heating.

Sometimes, synthetic hydrophobic monomers are used to regulate hydrogel properties for specific applications. They are chemically stronger compared to natural polymers; thus, their strength provides slow degradation rate but, on the other hand, there are more difficulties to dissolve. These two opposite characteristics should be balanced finding optimal design, for example to achieve desiderated controlled delivery.

Polymerization techniques can be used to obtain gels are, bulk, solution and suspension polymerization. Anyhow, the reaction starts in a system composed by monomer, initiator and cross-linker, as represented in figure 13. Then, the hydrogel mass needs to be washed to remove impurities left from the preparation process: non-reacted monomers, initiators, cross-linkers in excess and unwanted products obtained via side reactions.



*Fig. 13: Scheme of hydrogel preparation.*

### *USES*

Hydrogels use in the field of medicine and pharmacy seems to be the most successful and promising. Over 30 years of research in this range resulted in the common use of hydrogels as soft contact lenses, wound dressings, drug delivery systems and super-absorbents with a number of products being commercially available. This success can be related to the fact that some important properties of hydrogels, such as the ability to absorb aqueous solutions without losing shape and the mechanical strength, are commonly met in many natural constituents of a human body, like muscles, tendons, cartilage. In addition, hydrogels usually exhibit good biocompatibility in the contact with blood, body fluids and tissues, also evoking the use in tissue engineering.

Other important applications are in agriculture, sealing, food additives, hygienic products and biosensors.

## *HYDROGEL FUNCTIONALIZATION*

Functional groups at the end of the chain and along side chains of polymers ensure stable crosslinking of hydrogels and prevent their decay. Several physical and chemical strategies have been used for this purpose.

### *PHYSICAL CROSS-LINK*

Non-covalent interaction, such as ionic, hydrophobic or electrostatic interactions, crystallization, hydrogen bonding or combination of these, can be used in physical procedures to obtain final hydrogels. Amphiphilic block copolymers, proteins and peptides typically form network in this way, without the use of any potentially toxic chemical cross-linkers or initiators. Because of the weakness of the interactions, there is a limitation in their mechanical strength for some applications and then difficulties in the control over cell culture.

### *CHEMICAL CROSS-LINK*

Chemical strategies covalently couple reactive functional groups, using chain or step growth reactions. It is possible distinguish three principal methods: radical polymerization, Schiff base crosslinking and click chemistry.

### *RADICAL POLYMERIZATION*

Radical polymerization involves the formation of free radicals through the decomposition of an initiator by light, temperature or redox reaction. The successive reaction of radical propagation leads to the building of a polymer network. However, free radicals can be transferred to proteins, affecting their bioactivity, or biomolecules present in the cellular matrix, influencing cell viability. These reactions are exothermic and they can also produce local increase in temperature, shaking cell stability.

### *SCHIFF BASE CROSS-LINKING*

Schiff base crosslinking involves the reaction of macromolecules containing alcohol, amine or hydrazide functionalities with aldehydes to form a hydrogel network. Due to the mild reaction conditions, this process has been utilized to prepare cell-compatible hydrogels for cell encapsulation and controlled drug delivery applications.

### *CLICK CHEMISTRY*

Click chemistry represents a class of reactions for the synthesis of new compounds with different functionalities via heteroatom links. These reactions are driven by thermodynamic driving force ( $> 20 \frac{kcal}{mol}$ ), which is typically associated with the formation of carbon–heteroatom bonds. Molecules such as peptides, proteins, carbohydrates and lipids, can be assembled into large arrays or can be covalently attached to other biomolecules or synthetic scaffolds, such as polymers, viruses or dendrimers, to generate biohybrid materials with a diverse selection of new and improved properties. Click reactions have the following advantages:

- ◆ fast kinetics (that is shorter reaction time),
- ◆ high yield and high purity,
- ◆ regiospecificity,
- ◆ versatility,
- ◆ aqueous conditions,
- ◆ facile tuning of structural and mechanical properties using stoichiometry,

that make them useful for production of cell-compatible hydrogels. In effect, they offer a facile access to a broad range of polymeric materials that would be difficult to prepare otherwise.

From the initial publications, the area of click chemistry has turned into a highly productive area of research with exponential growth over the last few years.

The reaction involved in this category are:

-Huisgen cyclo addition;

-Diels-Alder reaction;

-Michael reaction;

-Thiol-ene reaction;

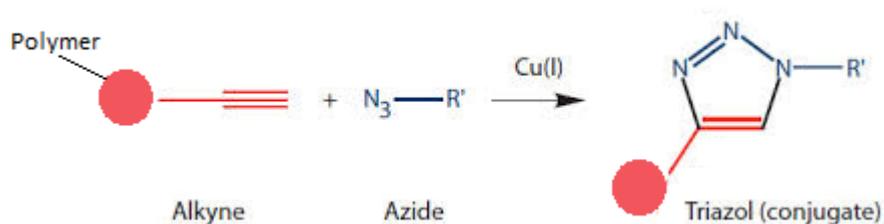
-Oxime reaction.

### *HUISGEN CYCLOADDITION*

This reaction is properly referred to as “click reaction”. It involves the interaction between an azide and an alkyne to form triazole, in presence of copper (I) catalyst. Both reagents are almost completely unreactive toward biological molecules, but on the other hand, there are limitations about alkyne homocoupling and difficulties removing residual heavy metal catalyst.

Hydrogels prepared in this way are based on triple bond functionalization of hyaluronic acid (HA), poly-acrylic acid (PAA) or polyethylene glycol (PEG) reacting with azide.

General scheme of this type of reaction is represented in figure 14.



*Fig. 14: Click reaction's scheme between generic polymer functionalized with triple bond and azide*

## DIELS-ALDER REACTION

Diels-Alder reaction is the addition of conjugated dienes to substituted alkenes to form substituted cyclohexenes. The dienophile (that is the substituted alkene) can be considered as simultaneously a nucleophile and electrophile. The reaction occurs under mild conditions and without initiator. The driving force is the conversion of two  $\pi$  bonds into two  $\sigma$  bonds, as indicated in figure 15.

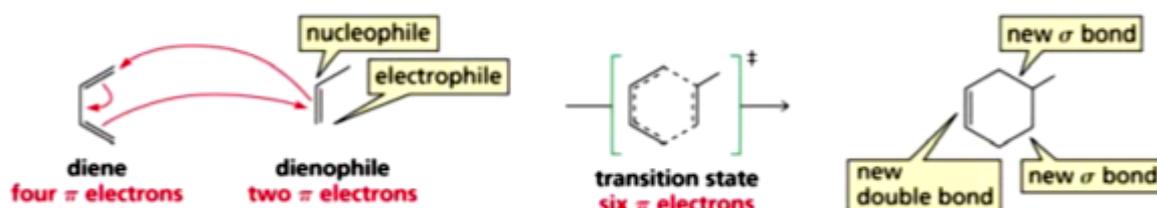


Fig 15:Diels-Alder mechanism reaction

## MICHAEL REACTION

Michael reaction involves two carbonyl components: an enolate and an  $\alpha,\beta$ -unsaturated, represented in figure 16.

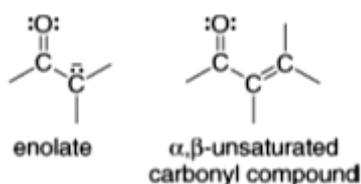


Fig. 16: Reagents of Michael addition.

In particular, a base removes the acidic proton between two carbonyl groups, forming the enolate. Then, the nucleophilic enolate attacks the  $\beta$  carbon of the  $\alpha,\beta$ -unsaturated carbonyl compound, obtaining a new carbon-carbon bond and a stabilized enolate. Protonation of the enolate forms the 1,4-addition product.

The mechanism is illustrated below (figure 17).

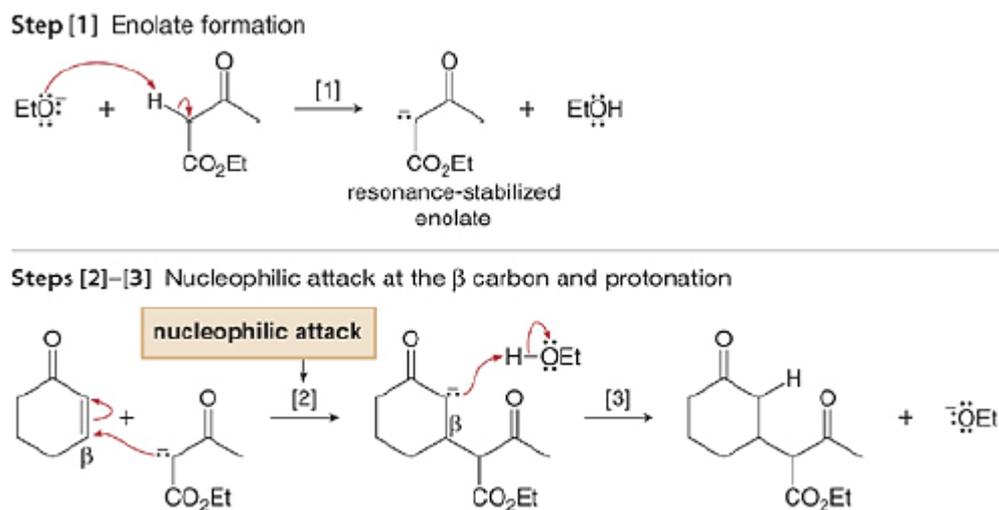


Fig. 17: Michael addition mechanism. The first step is enolate formation, The second is the nucleophilic attack by enolate and the third the protonation.

### THIOL-ENE REACTION

Thiol-ene reactions typically involve interaction between thiols and unsaturated functional groups, such as unactivated alkenes, maleimides and acrylates. They can proceed by free radical addition, Michael nucleophilic addition or a combination of these mechanisms depending on the reaction conditions. An example is the production of a hydrolytically degradable PEG hydrogel using thiol-acrylate mixed mode free radical photopolymerization.

### OXIME REACTION

This reaction involves aminoxy and aldehyde or ketone functional groups. It is classified as click system because of its fast reaction rate, compatibility to various molecules found in cell microenvironment and lack of catalyst. An example is the synthesis of cytocompatible PEG hydrogels functionalized with aminoxy groups and cross-linked with glutaraldehyde. By varying the polymer concentration and stoichiometric ratio of aminoxy to aldehyde, hydrogel mechanical properties and water content were modulated.

A review of the described reactions, with their operative conditions and applications, is reported in table 1.

Click reactions	Reacting functional groups	Reaction conditions <sup>221</sup>	Key features	Applications
CuAAC	Azide and alkyne	pH 4–12, reaction time < 1 h, Cu catalyst required	<ul style="list-style-type: none"> <li>- Bioorthogonal</li> <li>- Reversible</li> <li>- Difficulties with complete removal of cytotoxic Cu</li> </ul>	Cell encapsulation and delivery, drug delivery, 2D cell culture
Diels–Alder	Conjugated diene and substituted alkene	pH 5.5–6.5, reaction time < 8 h	<ul style="list-style-type: none"> <li>- No catalyst required</li> <li>- Longer reaction time than most of the other click reactions</li> </ul>	Cell encapsulation and release, controlled cargo delivery
Thiol-ene	Thiol and unsaturated functional group (radical mediated)	pH 6–8, reaction time < 1 h	<ul style="list-style-type: none"> <li>- Spatiotemporal control possible with select chemistries and using a photoinitiator</li> </ul>	Cell encapsulation, degradable 3D cell culture
Michael addition	Thiol and $\alpha,\beta$ -unsaturated carbonyl group	pH 6–8, reaction time < 30 min	<ul style="list-style-type: none"> <li>- No catalyst required</li> <li>- Reversible</li> </ul>	Cell encapsulation, controlled cargo delivery
Oxime	Aminoxy and aldehyde/ketone	pH 6–8, reaction time < 30 min	<ul style="list-style-type: none"> <li>- No catalyst required</li> </ul>	Cell encapsulation, protein immobilization

*Table 1: Click reactions with their key aspects*

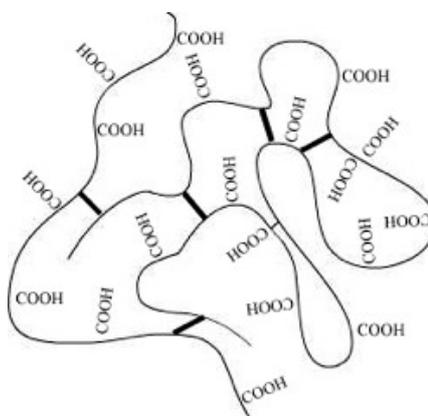
## HYDROGEL FORMULATION

After the preparation of required functionalized polymers, it is possible to proceed to hydrogels' synthesis. In this chapter, methods used to produce 3D polymeric networks are discussed.

Some of the synthesized hydrogels are also subjected to FTIR analysis, after preparation of the sample with KBr pellet technique. FTIR spectrum confirms the formation of expected products, emphasizing the adequateness of experimental procedures adopted.

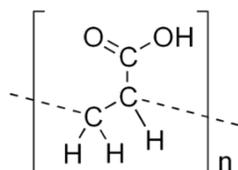
Referring to studies performed by Rossi et al. [14], biocompatible materials already known and used in biomedical and pharmaceutical areas are utilized for hydrogels formulation in this work. These materials are:

- ◆ Phosphate Buffer Saline (PBS). It is a water-based salt solution containing sodium phosphate, sodium chloride, potassium phosphate and minor amounts of carbonates and other sodium salts. The buffer nature helps to keep pH constant in hydrogel synthesis; the osmolality and ions concentration of the solution usually match those of human body.
- ◆ Carbomer 974P. It is a cross-linked poly-acrylic acid containing carboxyl groups (65%) that make it a ionizable molecule. The molecular weight is 1 million Da. The structure, shown in the figure 18, evidences the presence of a carboxyl group in each monomer.



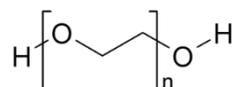
*Fig. 18: Carbomer structure*

- ◆ Poly-acrylic acid (PAA): polymer of acrylic acid, whose repeating units have a carboxyl group, as reported in figure 19, that can lose its proton thus acquiring a negative charge. This makes PAA a polyelectrolyte, with the ability to adsorb and retain water and swell to many times its original volume, which is useful characteristic to a functional hydrogel. In this work, PAA 35% w/w in aqueous solution is used.



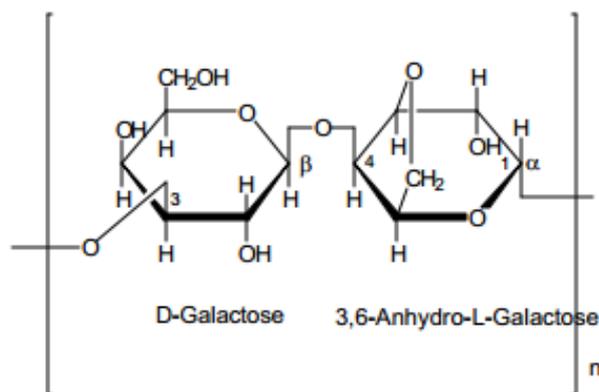
*Fig. 19: Chemical structure of PAA*

- ◆ Polyethylene glycol (PEG): hydrophilic non-degradable polymer of ethylene oxide. It lacks any protein binding sites and due to its hydrophilic and uncharged structure, it forms highly hydrated layers. It is characterized by excellent biocompatibility and low toxicity: in fact, PEG has been approved for several medical applications, including use as laxatives, solvents in liquid formulations, conjugates to therapeutic proteins, lubricants and hydrogels. Its repeating unit is represented in the figure 20 below. PEG with molecular weight equal to  $2000 \frac{g}{mol}$  was used in this synthesis.



*Fig. 20: Chemical structure of PEG*

- ◆ Agarose ultra-low gelling temperature. It is a purified linear galactan hydrocolloid isolated from agar or agar-bearing marine algae. Structurally, it is a linear polymer consisting of alternating D-galactose and 3,6-anhydro-L-galactose units, as illustrated in figure 21.



*Fig. 21: Chemical structure of agarose*

Agarose is used as gelling agent in biological applications, such as separation of nucleic acids via electrophoresis, formation of gel plates or overlays for cells in tissue culture and production of gel matrix. The gel point is the temperature at which an aqueous agarose solution forms a gel as it cools: agarose solutions exhibit hysteresis in the liquid-to-gel transition, that is, their gel point is not the same as their melting temperature. About this, it's used agarose with gel point in the range of 8-17° C.

### *AC-PEG FORMULATION*

The basic solution for hydrogel synthesis is prepared using PBS, Dulbecco's phosphate buffered saline solution.

Carbomer 974P due to its poly-acrylic molecular structure provides the carboxyl groups necessary in the esterification reaction for scaffold formation and PEG.

PAA pure is not added to the reaction system, because there is Carbomer 974P that performs the same task already.

Experimental procedure starts with dissolution of 0.23 g of PBS in 18 ml of distilled water. 100 mg of Carbomer 974P are added to the solution. It occurs under high rotation speed for 30 minutes, at room temperature. Then, 1.36 g of PEG are added and the solution is kept stirred for 45 minutes. Once mixing is over, the reaction system is removed from the mixer and left to stabilize for 30 minutes; after, pH is carried to 7.4

adding NaOH 1 N. The obtained system was modified in order to introduce TEMPO derivatives, following the target of the synthesis of a hydrogel acting as contrast agent, as discussed in the section of experimental hydrogel synthesis.

## CYCLODEXTRINS

Cyclodextrins are naturally occurring oligosaccharides, macrocycles composed of  $\alpha$  (1,4)-glucopyranose subunits and were first reported in 1891. They are obtained by the enzymatic degradation of starch, a process that yields linear and cyclic oligomers of glucose, referred to as *dextrins* and *cyclodextrins* respectively. There are three particularly important cyclodextrins:  $\alpha$ -cyclodextrin, which contains 6 D-glucose units,  $\beta$ -cyclodextrin with 7 and  $\gamma$ -cyclodextrin composed of 8 sugar units (Fig 22).

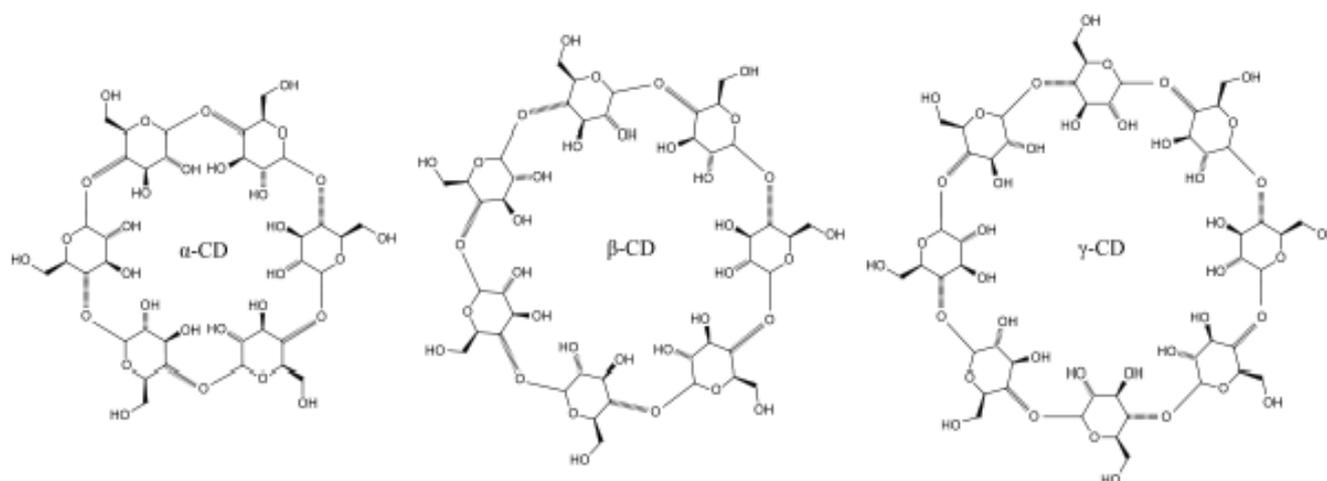


Fig. 22: Chemical structure of  $\alpha$ ,  $\beta$  and  $\gamma$  cyclodextrins

As a consequence of the  $4C_1$  chair conformation of the glucopyranose units, the hydroxyl functions are oriented to the external part of the cone with the primary hydroxyl groups of the sugar at the narrower edge of the cone (“primary face”) and the secondary hydroxyl groups at the wider edge (“secondary face”). The ring takes the shape of a truncated cone (Fig. 23).

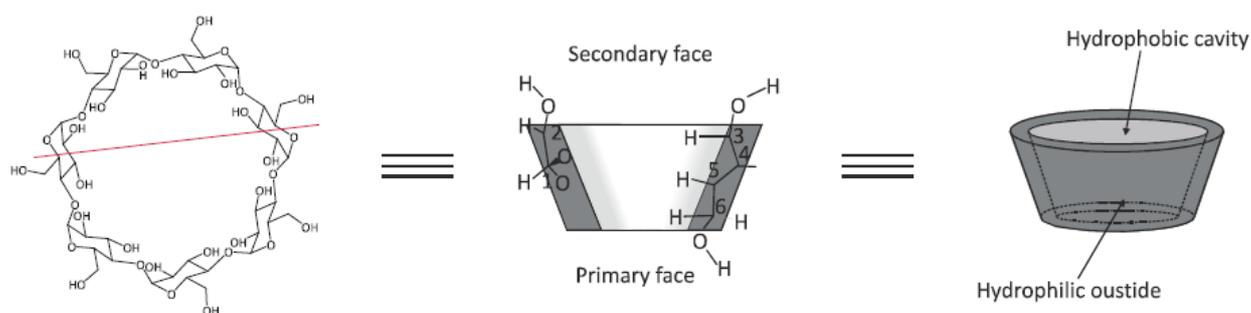
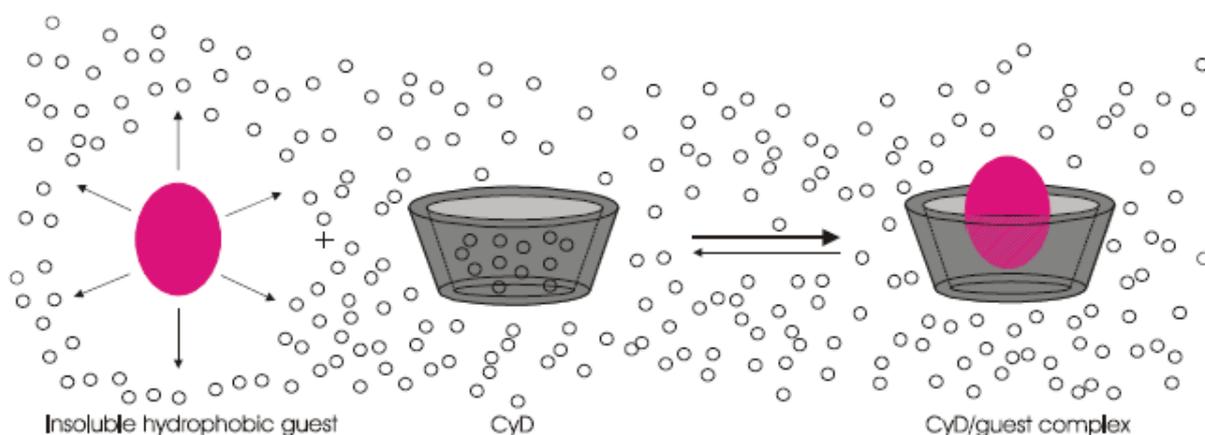


Fig. 23: Morphology of CDs

As a result of the OH groups deriving from the glucoses, the hydrophilic external surface of cyclodextrins shows polar properties, allowing the formation of H-bonds with protic solvents, such as water. The inner cavity, instead, is non-polar, and it's able to bind different hydrophobic guests. In this case, hydrophobic interactions are the main source of the binding strength. The lipophilic cavity provides a microenvironment into which appropriately sized non-polar moieties can enter to form inclusion complexes. No covalent bonds are broken or formed during the formation of the inclusion complex. The main driving force of this process is the release from the cavity of water molecules rich in enthalpy (Fig. 24).



*Fig. 24: Schematic representation of inclusion complex formation*

Therefore the binding of the guest molecule within the host CD is not fixed, but it is rather a matter of dynamic equilibrium. The binding strength depends on the stability of the complex formed, which means how well the “ host-guest “ complex fits together, and on specific local hydrophobic interactions between surface atoms. Water is typically the solvent of choice for the formation of these CD-complexes.

## SAFETY AND BIOCOMPATIBILITY OF CDs

The natural  $\alpha$ -CD and  $\beta$ -CD, unlike  $\gamma$ -CD, cannot be hydrolyzed by the human salivary and pancreatic amylases. However, both  $\alpha$ - and  $\beta$ -CD can be fermented by the intestinal microflora. CDs are both large (MW ranging from almost 1000 to over 2000 Daltons) and hydrophilic with a significant number of H-donors and acceptors. As a consequence, they are poorly absorbed by the gastrointestinal tract in their intact form and are considered non-toxic at low to moderate oral dosages.

These molecules possess a cage-like supramolecular structure, comparable with the structures of crown ethers, cryptands, spherands, cyclophanes, and calixarenes. However, it took 50 years to establish the molecular structure of CDs. Owing to their capability to form inclusion complexes with a variety of guest molecules, CDs are considered as the most important supramolecular host family among all the ones mentioned above. They can form complexes with various types of molecules including inorganic, organic, or organometallic, that can be radical, cationic, anionic, or neutral molecules. This phenomenon bears the name of "molecular recognition". In addition, the properties of the molecules forming the complexes with CDs can be modified significantly. As such, a large number of scientists have attempted to elaborate and evaluate different CD derivatives that are able to complex a variety of drugs, enhancing by this way their *in vivo* solubility and activity in drug delivery systems, using various routes of administration. Moreover, a large number of publications describe CD uses in other fields such as foods, textile, cosmetics, or agriculture.

Lipophilic CD derivatives, such as the methylated CDs, are to some extent absorbed from the gastrointestinal tract into the systemic circulation and have been proved to be toxic after parenteral administration. Some differences are anyhow present among the different types of CDs: for instance, in the case of  $\beta$ -CD, it cannot be parenterally administered due to its low aqueous solubility and adverse effects (e.g. nephrotoxicity) whereas the metabolism of  $\gamma$ -CD closely resembles that of starch and linear dextrans. Oral administration of 8 g of  $\gamma$ -CD or 8 g maltodextrin to humans did not reveal any differences in gastrointestinal tolerance of these two oligosaccharides.

### *CDs AS CARRIERS FOR ANTIOXIDANTS MOLECULES*

One of the biggest obstacle to overcome during the administration of ROS Level Modulators (RLM) is the poor solubility of antioxidants. The design and production of polymeric vectors as biocompatible carriers, in a stable chemical form, with sufficient concentration and with reduced or, possibly, null adverse effects, is highly valuable.

These vectors should combine:

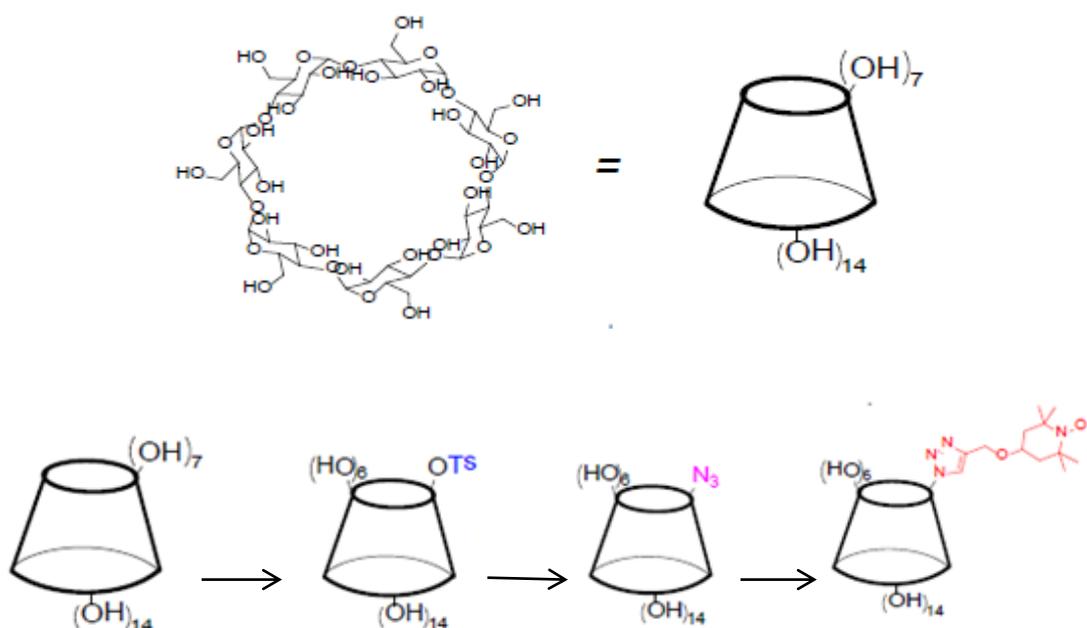
- High and selective RLM delivery capability to the target tissue
- Possible intrinsic ROS scavenging activity
- Detectability by advanced diagnostic techniques
- Reduced bioaccumulation in the human body
- Biocompatibility

### *PARAMAGNETIC CYCLODEXTRIN*

The introduction of a persistent radical (TEMPO) in a biocompatible carrier (like CD) allows the production of a paramagnetic device with dual benefits:

- Introducing probes for both further characterization of nanomaterials by means of Electron Spin Resonance (ESR) and detection of nanoparticles *in vitro* and *in vivo*;
- Introducing organic paramagnetic labels to rapidly vehicle nanoparticles in specific ill areas by application of mild magnetic fields.

With this aim, referring to some previous studies on this topic, a new paramagnetic cyclodextrin was synthesized by Prof Punta's group [5] using a ratio TEMPO/CD = 1:1, in order to obtain one TEMPO moiety for each CD, following the steps below (scheme 1):



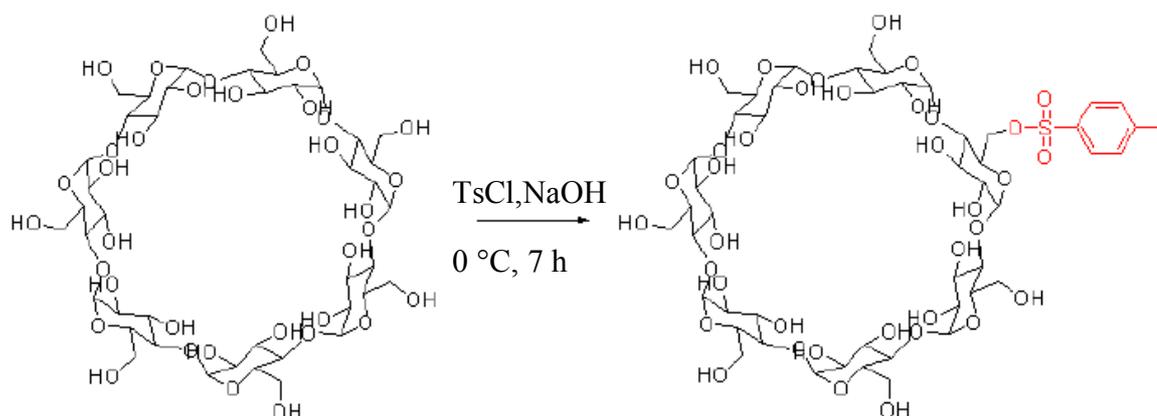
*Scheme 1: Scheme of the synthesis of paramagnetic  $\beta$ -cyclodextrins [5]*

The link between the CD and the nitroxyl radical is given by a click reaction. To obtain the Huisgen cycloaddition, an azide group is introduced into a CD and an alkyne group to TEMPO in order to promote this molecular interconnection.

In details the functionalization of a CD with the azide group requires an intermediate step: initially one C6 position of the CD is activated by a tosyl group (scheme 2) that is substituted in turn by an azide group (scheme 3).

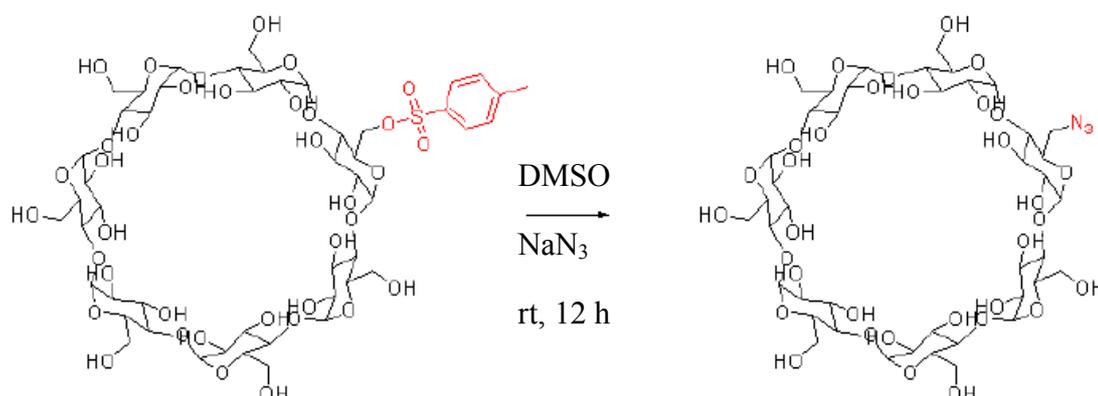
- **Tosylation of  $\beta$ -CD**

The tosylation of CDs expects a sulfonate ester bond between tosyl group and CD-OH. Sulfonyl chloride converts the alcohol into its tosylate ester by the standard nucleophilic substitution sequence, where the OH reacts as a nucleophile, attacking the electrophilic center of tosylate, displacing a chloride ion and finally giving a sulfonate ester.



*Scheme 2: Tosylation of  $\beta$ -CD*

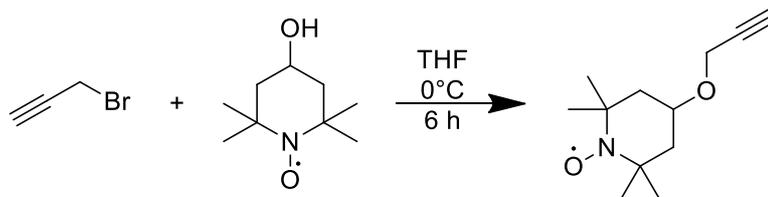
- **Substitution of Tosyl group by azide group**



*Scheme 3: Mono 6-(p-toluenesulfonyl)-6-deoxy- $\beta$ -cyclodextrin is converted in mono 6-azido-6-deoxy- $\beta$ -cyclodextrin by  $S_N2$  reaction*

- **Synthesis of Propargyl –TEMPOL**

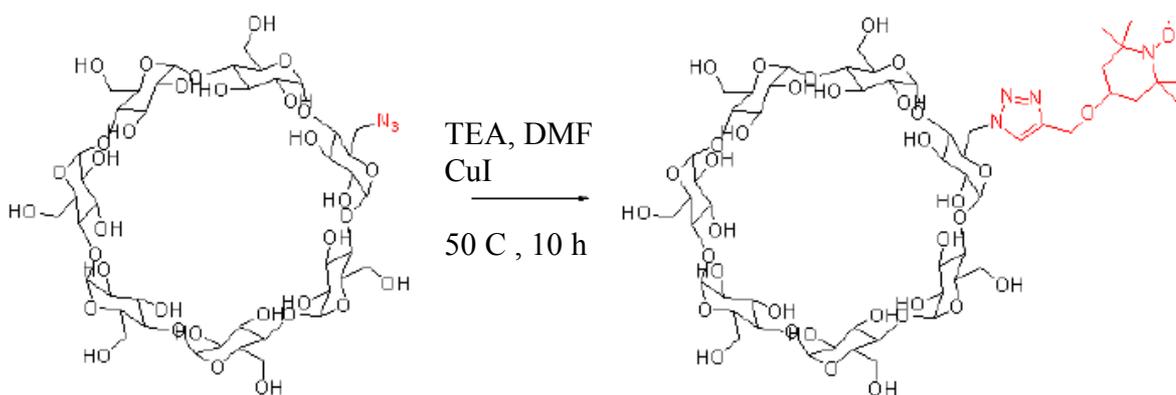
With regards to the derivatization of TEMPOL, the propargyl moiety can be easily introduced by a nucleophilic reaction between the OH group and the propargyl bromide, with the release of a HBr molecule. (scheme 4)



*Scheme 4: Preparation of TEMPOL-propargyl intermediate*

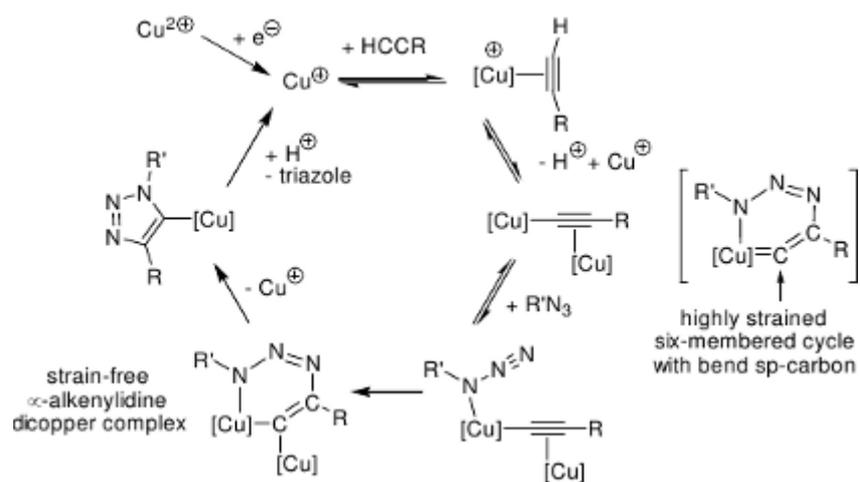
- **Click reaction**

The final step follows the classical Huisgen's mechanism, with the formation of the triazole, used as a stable linker between the CD and TEMPOL, which decorates the macrocycle like a pendant. (Scheme 5)



*Scheme 5: Click reaction between CD and TEMPOL*

The Huisgen's mechanism is represented in the scheme below:



Scheme 6: Huisgen's reaction mechanism

# Results and discussion

## AIM OF THE THESIS

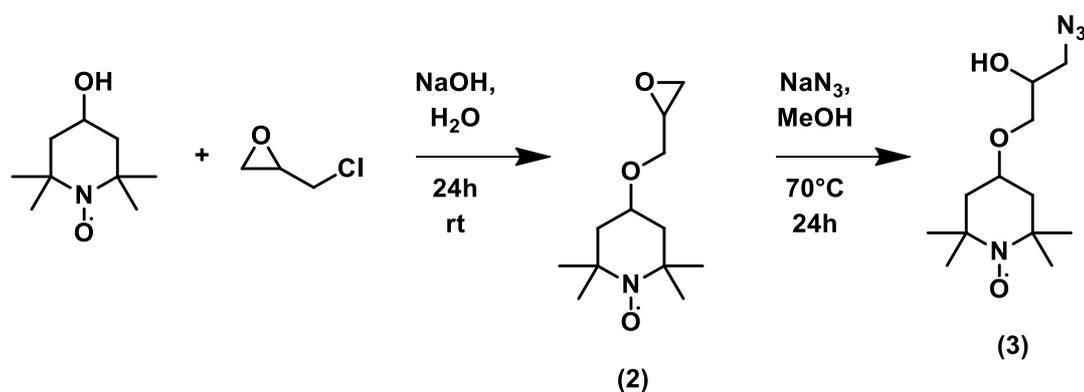
The aim of this thesis is the synthesis of new hydrogels as contrast agents media for MRI. For this purpose, hydrogels are labelled by TEMPOL, that is a paramagnetic spin label and, hence, it can be detected by MRI. These hydrogels are particularly attractive because they are biocompatible and injectable. The effectiveness of our material is shown by the evaluation *in vitro* and at Mario Negri research institute. In addition, we exploited the possibility to form  $\beta$ -CD-TEMPOL hydrogels, synthesized by Prof. Punta's group (Caglieris, F.; Melone, L.; Canepa, F. et al, *RCS Adv.*,**2015**, (5),76133-76140).

## SYNTHETIC STRATEGIES FOR THE FUNCTIONALIZED POLYMERS

Before proceeding to the hydrogels synthesis, it is necessary to prepare the required functionalized polymers. These ones are based on PAA and PEG. In all cases, we tried two different approaches to functionalize the polymer with TEMPOL: on one hand we took advantage of click chemistry, on the other hand we exploited alternative syntheses.

- **Synthesis of PAA-TEMPOL**

The click reaction requires the presence of the azide group and the triple bond. Regarding the synthesis of PAA-TEMPOL, the azide group is provided by TEMPOL-azide, which synthesis is made up of the following steps (scheme 7):



Scheme 7: synthesis of TEMPOL-azide

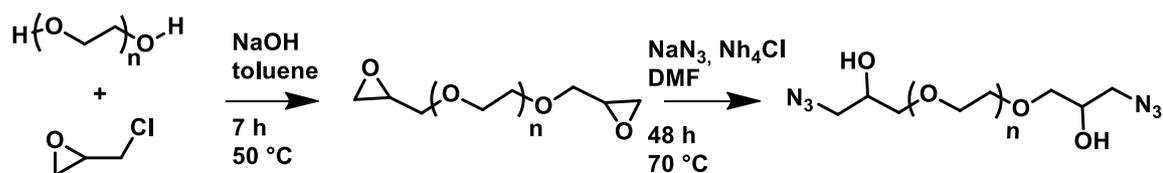
The triple bond is provided by Propargyl-PAA (1).

- **Alternative synthesis of PAA-TEMPO**

Ester bonds and Amide bonds are alternative linkers of click chemistry. They allow a covalent connection between the functional groups of the polymers and the TEMPO moiety. Amine-TEMPO (commercially available) reacts with PAA, affording the PAA-TEMPO connected through the amide linker. TEMPOL reacts with PAA, affording PAA-TEMPO, bond through the ester linker. This last synthetic strategy, however, shows the main drawback of the cleavable ester bond *in vivo*.

- **Synthesis of PEG-TEMPO**

Regarding the synthesis of PEG-TEMPO, the azide group is provided by azide-hydroxyl-PEG, which synthesis is made up of the following steps (scheme 8):



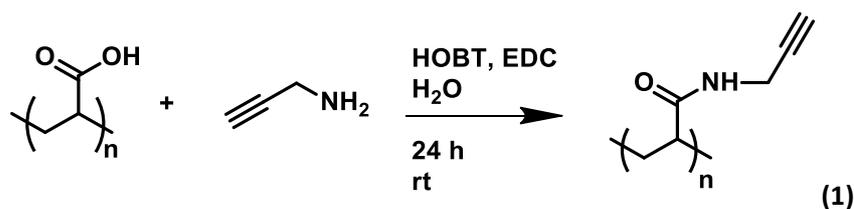
Scheme 8: synthesis of azide-hydroxyl-PEG

The triple bond is provided by propargyl-TEMPO, synthesized by Prof. Punta's group (Cagliaris, F.; Melone, L.; Canepa, F. et al, *RCS Adv.*, **2015**, (5), 76133-76140).

- **Alternative synthesis of PEG-TEMPO**

In order to synthesize PEG-TEMPO, without the contribution of click chemistry, two strategies are followed. In the epoxide-TEMPO strategy, the nucleophilic PEG reacts with epoxide-TEMPO in a  $S_N2$  type of reaction. In the epoxide-PEG strategy, the nucleophilic TEMPOL reacts with epoxide-PEG with the same mechanism.

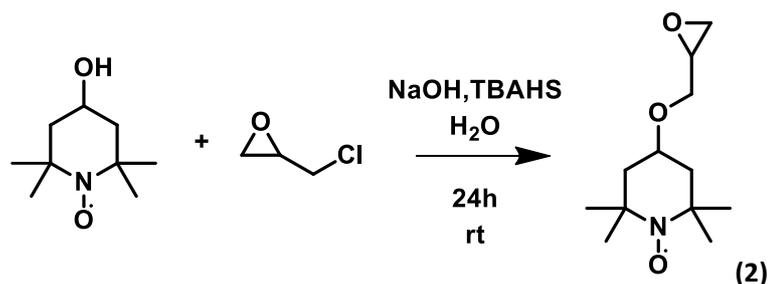
## SYNTHESIS OF PROPARGYL PAA (1)



In this synthesis an aqueous solution of PAA at 35% w/w, characterized by a density of 1.14 g/ml, is used. Propargylamine is in hydrochloride form and its density is equal to 0.86 g/ml. The procedure is referred to literature, in particular based on Ossipov's works (Ossipov D. et al. (2010) and Ossipov D. et al. (2013)). The reaction requires the use of HOBt and EDC, whose amounts are calculated considering molar ratio 1:1 with respect to propargylamine.

PAA (1 g, 1 eq.) is dissolved in 15 ml of distilled water. Propargylamine (253 mg, 0.2 eq.) is added to the solution. HOBt is dissolved in 15 ml of a solution composed by H<sub>2</sub>O and CH<sub>3</sub>CN (1:1) at about 50°C because of its insolubility at room temperature and added dropwise to the mixture (which is at room temperature). EDC powder (800 mg) is added. The mixture is stirred for about 24 hours at room temperature. The system is subsequently dialyzed. After dialysis, the solution with desired product is frozen at -21°C and lyophilized.

## SYNTHESIS OF TEMPO- EPOXIDE (2)

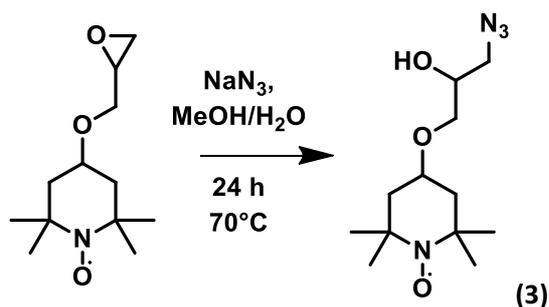


A solution of water and NaOH is prepared, dissolving 4.5 g of NaOH in 5 ml of water. 2 ml of this solution are poured in a flask with the other reactants: epichlorohydrin (18 ml,  $\rho=1.18\text{g/ml}$ , 20 eq), Tempol (2 g, 1 eq) and tetra-butyl-ammonium-hydrogen-sulfate (0.23 g, 0.05 eq). Then the solution is stirred at 30 C for 24 hours. Hence it is cooled down with ice. The organic phase is extracted with diethyl ether and it is dried over anhydrous sodium sulphate. The product is purified through a silica gel column (hexane/ethyl acetate 2:1) and it appears as a dark red liquid. A yield equal to 26% is reached.

<sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$  7.12 (bs, 1H), 3.69 (dd, 1H,  $J= 11, 2.7$  Hz), 3.60 (m, 1H), 3.24 (dd, 1H,  $J = 11, 6.6$  Hz), 3.04 (m, 1H), 2.70 (t, 1H,  $J= 5.1$  Hz), 2.52 (dd, 1H,  $J= 5.1, 2.7$  Hz), 1.86 (dd, 2H,  $J= 12, 3.6$  Hz), 1.24 (t, 2H,  $J= 11$  Hz), 1.05 (d, 12H,  $J=11$  Hz).

GC/MS :  $t_r = 20.437$  min

### SYNTHESIS OF TEMPO-AZIDE (3)



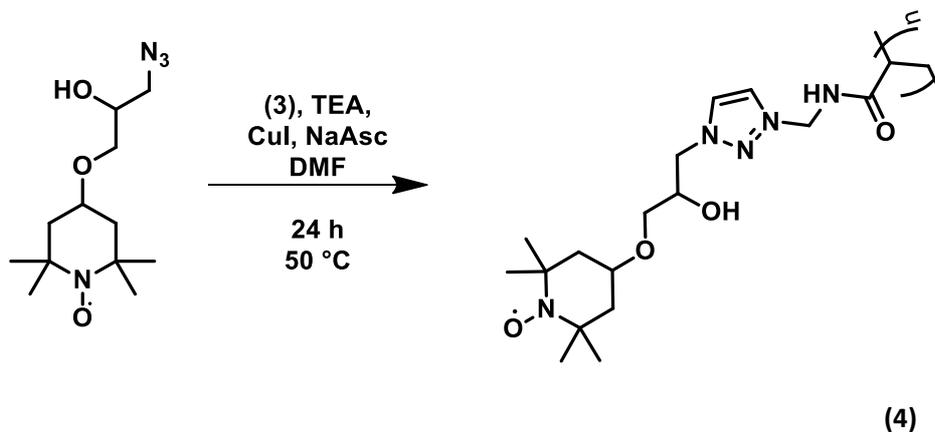
4.2 g of sodium azide (2 eq) and 5.6 g of Tempo-epoxide (1 eq) are dissolved in 15 ml of methanol and 15 ml of water. The mixture is heated for 24 hours at 65 °C. A liquid – liquid extraction with ethyl acetate and water is subsequently made after removing MeOH under vacuum. The organic phase, containing the product and ethyl acetate, is washed with brine three times, dried over sodium sulphate and under vacuum. The crude product is finally purified through a silica gel column (hexane/ethyl acetate 1:1) and it appears as a dark red liquid. A yield equal to 4.5% is reached.

GC/MS :  $t_r = 23.587$  min

$^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ )  $\delta$  5.31 (s, 1H), 3.90 (tt,  $J = 6.1, 4.6$  Hz, 1H), 3.66 – 3.54 (m, 1H), 3.47 (qd,  $J = 9.4, 5.3$  Hz, 2H), 3.35 (dd,  $J = 5.5, 4.2$  Hz, 2H), 1.91 (dq,  $J = 12.9, 3.6$  Hz, 2H), 1.53 – 1.38 (m, 2H), 1.21 (s, 6H), 1.15 (s, 6H).  $^{13}\text{C}$  NMR (101 MHz,  $\text{CDCl}_3$ )  $\delta$  71.5, 69.7, 69.5, 59.6, 53.5, 44.4, 31.8.

GC/MS :  $t_r = 23.587$  min

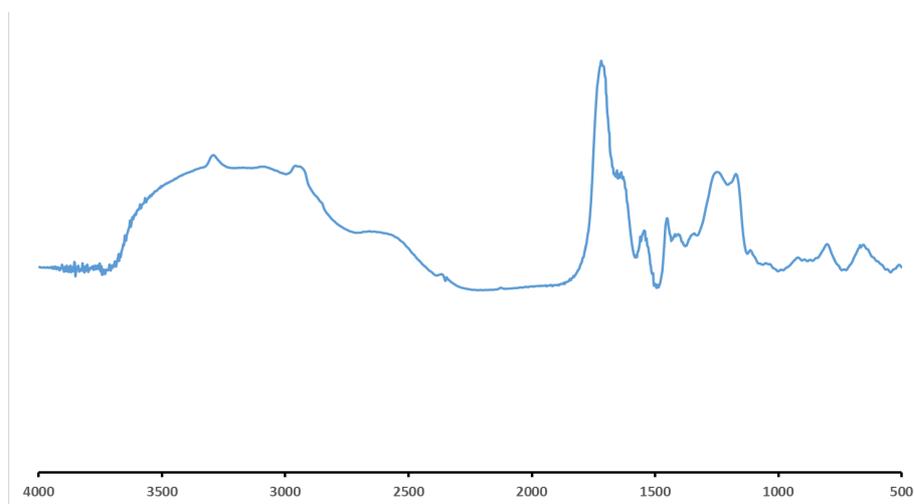
## SYNTHESIS OF PAA – TEMPO (4)



This procedure is based on Melone's work (Cagliaris, F.; Melone, L.; Canepa, F. et al, *RCS Adv.*,**2015**, (5),76133-76140).

500 mg of PAA (1 eq) are dissolved in 4 ml of DMF. 210 mg of Tempo-azide (0.15 eq) are dissolved in 1.5 ml of DMF. Then, PAA and Tempo-azide solutions are mixed together and sodium ascorbate (6 mg, 0.03 eq) is added to the solution. Separately, CuI (6 mg, 0.03 eq) is dissolved in 1 ml of DMF and 0.01 ml of EtN<sub>3</sub>. This solution is added to the previous mixture and stirred at 50 °C for 24h. The product is purified through dialysis and finally lyophilized. 100 mg of PAA-TEMPO are obtained and the product appears as a white solid.

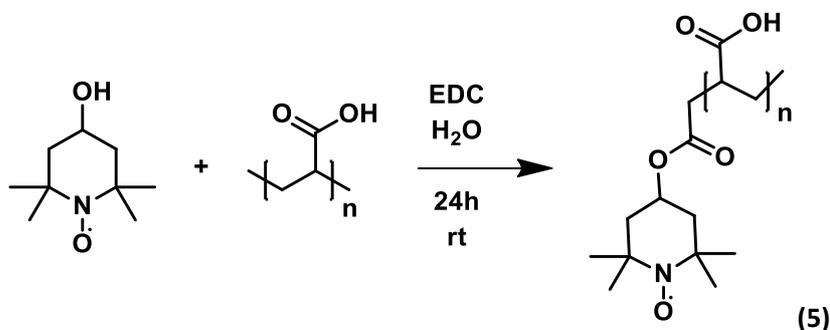
FTIR analysis: KBr pellet technique.



### ALTERNATIVE SYNTHESIS OF PAA-TEMPO

In order to obtain a polymer based on PAA and tagged by TEMPOL, we followed two different synthetic approaches.

#### 1) Ester linker

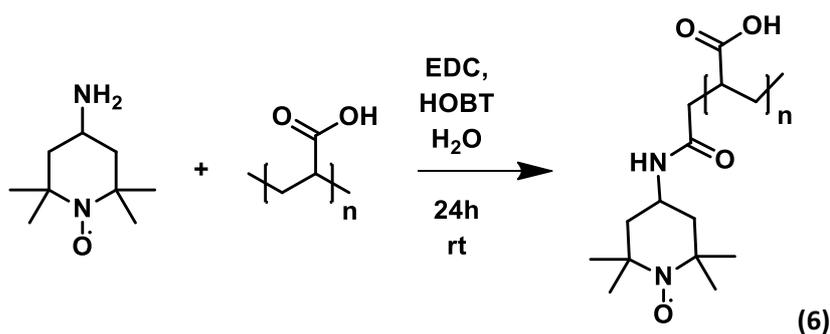


In this synthesis an aqueous solution of PAA at 35% w/w, characterized by a density of 1.14 g/ml, is used. The procedure is referred to previous Ossipov's work (Ossipov D. et al. (2010) and Ossipov D. et al. (2013)). The reaction requires the use of EDC. Its amount is calculated considering molar ratio 1.25 with respect to TEMPOL.

PAA (144 mg, 2 eq.) is dissolved in 15 ml of distilled water, then TEMPOL (172 mg, 1 eq.) is added to the solution. After addition of EDC powder (194 mg), the mixture is stirred for 24 hours at room temperature. The system is dialyzed and, after that, the solution with desired product is frozen at -21°C and lyophilized.

A white solid (0.43 g) was obtained but the NMR analysis shows that it was not the right product.

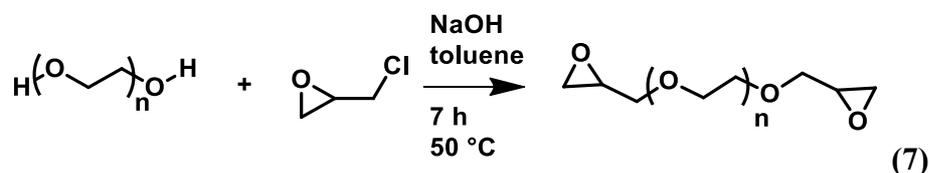
## 2) Amide linker



In this synthesis an aqueous solution of PAA at 35% w/w, characterized by a density of 1.14 g/ml, is used. The procedure is referred to previous Ossipov's work (Ossipov D. et al. (2010) and Ossipov D. et al. (2013)). The reaction requires the use of HOBT and EDC, whose amounts are calculated considering molar ratio 1.25:1.25 with respect to Amino-Tempo, commercially available.

PAA (165 mg, 2 eq.) is dissolved in 15 ml of distilled water. Amino-Tempo (200 mg, 1 eq.) is added to the solution. HOBT (190 mg) is dissolved in a solution (5ml) composed by H<sub>2</sub>O and CH<sub>3</sub>CN (1:1) at 50°C, because of its insolubility at room temperature, and added dropwise to the mixture (which is at room temperature). EDC powder (270 mg) is added and the mixture is mechanically stirred for 24 hours at room temperature. The system is dialyzed and, after dialysis, the solution with desired product is frozen at -21°C and lyophilized.

## SYNTHESIS OF PEG-EPOXIDE (7)

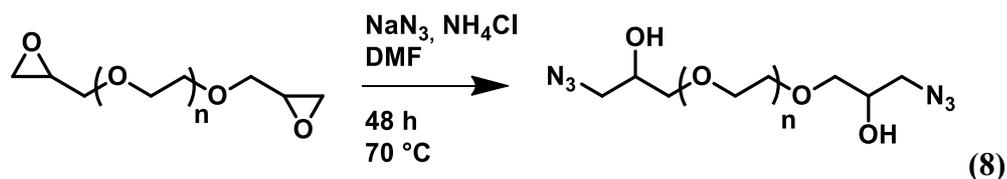


This procedure refers to Teodorerescu's work ( Teodorescu,M.; Cursaru, B.; Stanescu,P. et al., *Polym.Adv.Techn.*,**2009**, (20), 907-915).

PEG 2000 (2.0 g, 1 mmol) is dissolved in 8 mL of toluene. Epichlorohydrin ( 1.175 mL,  $\rho = 1.18$  g/mL, 15 eq. ) is added, followed by sodium hydroxide (0.6 g , 15 eq.) is added. The mixture is stirred at 50 °C for 7 hours. The product is extracted with water. The aqueous phase, containing PEG-EP and water, is washed with brine and extracted from dichloromethane (DCM). The organic phase is dried over anhydrous  $\text{Na}_2\text{SO}_4$ , filtered, concentrated by rotary evaporation and precipitated into cold diethyl ether. The product (1.45 g ) appears as a white solid powder.

$^1\text{H}$  NMR (400 MHz, Chloroform-*d*)  $\delta$  3.78 (dd,  $J = 11.7, 3.1$  Hz, 1H), 3.64 (s, 83H), 3.44 (dd,  $J = 11.6, 5.9$  Hz, 1H), 3.16 (ddt,  $J = 5.8, 4.1, 2.9$  Hz, 1H), 2.79 (dd,  $J = 5.1, 4.1$  Hz, 1H), 2.61 (dd,  $J = 5.0, 2.7$  Hz, 1H).

### SYNTHESIS OF AZIDE-HYDROXYL PEG (8)

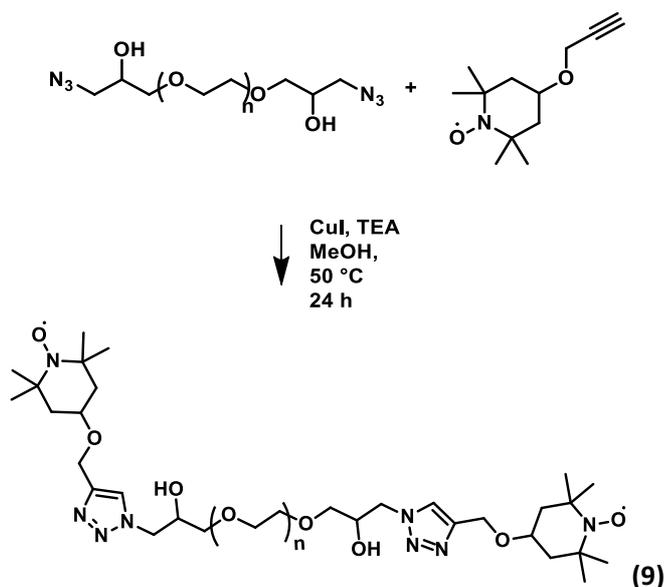


This procedure refers to Zhang's work (Zhang,B.; Hang,H.; Elupula,R. et al., *Macromolecular Rapid Communications*,**2014**, (35), 146-151).

The synthesis of PEG–OH–N<sub>3</sub> is accomplished by the ring-opening of (7) with NaN<sub>3</sub>. PEG-EP (0.5 g, 1 eq.), NaN<sub>3</sub> (73 mg, 5 eq.) and NH<sub>4</sub>Cl (120 mg, 10 eq.) are dissolved in 2.5 mL of DMF. The reaction is stirred at 70 °C for 48 h. The mixture then is filtered to remove the salt, and the DMF is removed under vacuum. The crude product is dissolved in dichloromethane and washed with water three times, to remove any residual salt. The resultant organic solution is dried over sodium sulfate, filtered, concentrated under vacuum and then precipitated into cold diethyl ether. 0.14 g of azide-hydroxyl peg are obtained. The product is a white powder.

<sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  3.95 (q, J = 5.1 Hz, 2H), 3.64 (s, 180H), 3.58 – 3.38 (m, 5H), 3.37 – 3.25 (m, 6H), 1.21 (t, J = 7.0 Hz, 1H).

## SYNTHESIS OF TEMPO TERMINATED PEG: PEG-TEMPO (9)



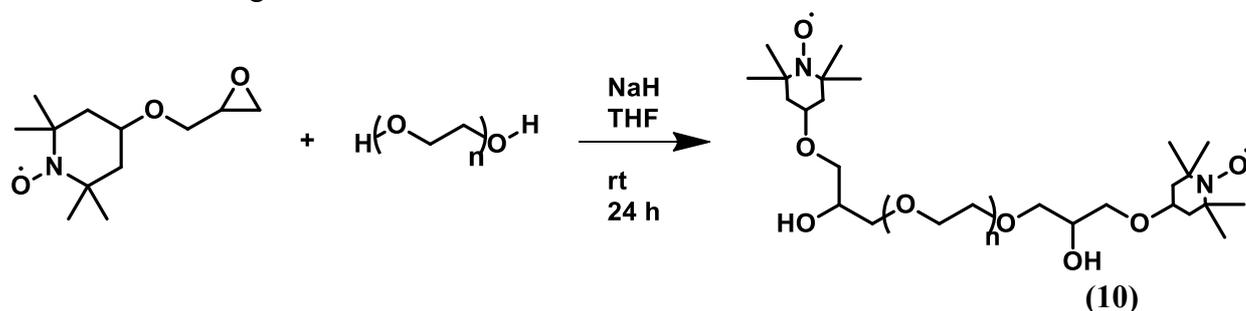
This procedure refers to Melone's work (Caglieris, F.; Melone, L.; Canepa, F. et al, *RCS Adv.*,**2015**, (5),76133-76140).

In a two necked flask, **(8)** (0.14 g., 1eq.) and propargyl-TEMPO (0.035 g, 3 eq.) are dissolved in 5 mL of methanol. CuI (20 mg, 2 eq.) and a few drops of triethylamine are added to the solution. The reaction is stirred under nitrogen atmosphere for 24 h at 50 °C. The final product is dialyzed to remove copper particles and then lyophilized. Peg-TEMPO (0.16 g) appears as a solid grey product.

$^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ )  $\delta$  7.75 (s, 1H), 4.48 (m, 6H), 4.19 (m, 3H), 3.64 (s, 86H), 2.17 (m, 2H), 1.26 (s, 9 H).

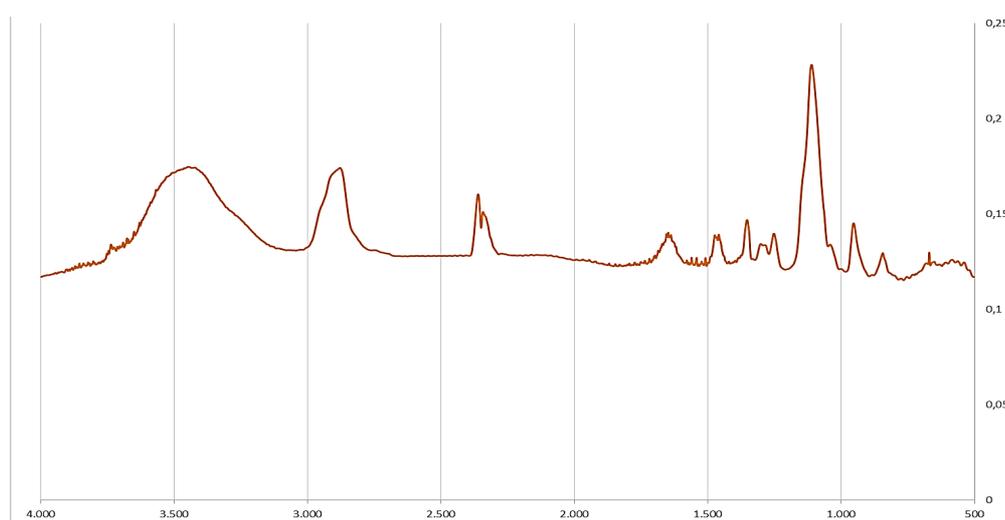
## EPOXIDE-TEMPO STRATEGY

In order to obtain a polymer based on PEG 2000 and tagged by TEMPOL, we followed two different strategies.

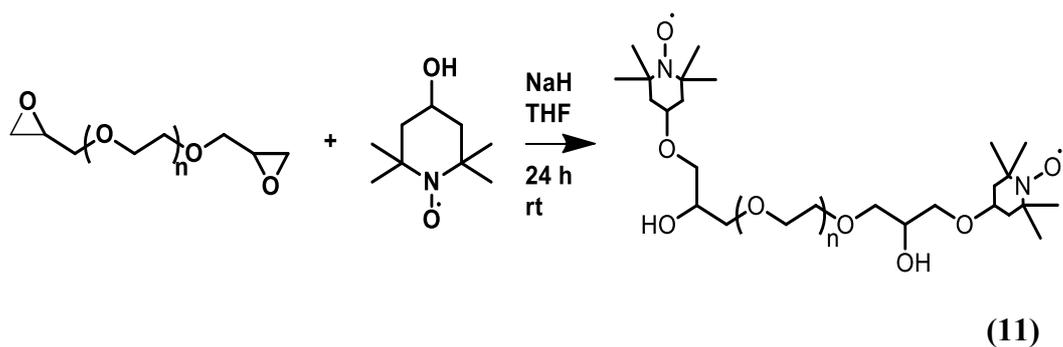


0.26 g of PEG 2000 (1 eq.) and 12 mg of sodium hydride (4 eq.) are dissolved in 10 ml of THF. After 1 hour, Tempo-epoxide (0.07g, 3 eq.) is added. The solution is mixed for 24 hours at room temperature. The organic phase is then extracted with DCM and water. Brine is also added to avoid emulsion. Finally the product is dried over sodium sulfate and the solvent is removed under vacuum. It is then precipitated into cold diethyl ether and filtered with a Buchner funnel. The product ( 220 mg) is a grey solid.

FTIR analysis: KBr pellet technique.



### EPOXIDE-PEG STRATEGY



43 mg of TEMPOL (0.05 mmol, 1 eq.) and 9 mg of sodium hydride (0.25 mmol, 5 eq.) are dissolved in 5 ml of THF. After 1 hour, 0.1 g of PEG 2000-epoxide (1 eq.) is added. The solution is stirred at ambient temperature for 24 hours. Liquid  $\text{NH}_4\text{Cl}$  is added and THF is removed under vacuum. The product is extracted from DCM and dried over sodium sulfate, then DCM is removed under vacuum. The product is precipitated into cold diethyl ether and filtered through a Buchner funnel. It appears as a white powder and its amount is equal to 60 mg).

Given that NMR analysis shows a 50% conversion, no further investigations are performed.

## *EXPERIMENTAL HYDROGEL SYNTHESIS*

Hydrogels are prepared starting from AC-PEG formulation and TEMPOL, polymers labelled by TEMPO and  $\beta$ -CD-TEMPO. The experimental procedures are listed below:

### *TEMPOL FUNCTIONALIZED HYDROGEL*

In order to make hydrogels grafted by TEMPOL two different pathways can be followed. The first one consists on dissolving TEMPOL in the water-PBS solution, and diluting this last one with the ac-peg formulation after microwaves irradiation. The second one consists on directly dissolving TEMPOL in the AC-PEG formulation. In this mixture, agarose is added and the system can be subjected to microwaves irradiation.

#### *First Pathway*

Two dilutions are prepared, in order to have two different concentrations of TEMPOL in the water-PBS solution (20 mM and 60 mM).

17 mg of TEMPOL are dissolved in 5 ml of water-PBS solution (20 mM, sample **A**).

51 mg of TEMPOL are dissolved in 5 ml of water-PBS solution (60 mM, sample **B**).

To 6 ml of the AC-PEG formulation, 30 mg of agarose powder are added and this system is irradiated with microwave radiation at 500 W until boiling, for a time usually between 30 seconds and 1 minute, and electromagnetically heating up to 80 °C. At this point the gel is liquid and condensation reactions begins.

When the temperature achieves 50 C, 500  $\mu$ l are taken and placed in Eppendorf where 500  $\mu$ l of the sample **A** (20 mM ) are placed.

The same procedure is done for the sample **B** (60 Mm).

Gelatinization is complete after 20 minutes.

## *Second Pathway*

Two samples are prepared, in order to have two different concentrations of TEMPOL in the AC-PEG formulation (10 mM and 30 mM).

### Sample C (10 mM)

8.5 mg of TEMPOL are dissolved in 5 ml of AC PEG formulation. Then, 25 mg of agarose are added. The system is subjected to electromagnetic stimulation (500 W) for a minute. After irradiation, when the temperature achieves 50 °C, 1 ml is taken and placed in Eppendorf. Gelatinization occurs after 10 minutes.

### Sample D (30 mM)

8.5 mg of TEMPOL are dissolved in 5 ml of AC PEG formulation. Then, 25 mg of agarose are added. The system is subjected to electromagnetic stimulation (500 W) for a minute. After irradiation, when the temperature achieves 50 °C, 500 µl are taken and placed in steel cylinders in order to confer cylindrical shape to the hydrogels, and left at rest for about 45 minutes until complete gelatinization. Hydrogels are finally removed from the cylinders and put in 12 multi-well plate. In this plates, distilled water is added in order to submerged hydrogels and remove residual air ball which can be formed during the synthesis because of electromagnetic stimulation and subsequently gelation.

## *β-CD-TEMPO FUNCTIONALIZED HYDROGEL*

In order to establish the possibility to make hydrogels including β-CD-Tempo, 10 mg of β-CD-Tempo are mixed in 5 ml of the ac-peg formulation and sonicated for 10 minutes at 40 C. A sonicator cycle is necessary to make CD soluble in water, because CDs aren't soluble in water at ambient temperature or higher. Dissolving 10 mg of β-CD-TEMPO in 5 ml of water, it is not possible to obtain a completely miscible mixture without ultrasound. Then, 25 mg of agarose are added to the system of β-CD-TEMPO and AC PEG. The system is subjected to electromagnetic stimulation (500 W) for a minute. After irradiation, when the temperature achieves 50 C, 1 ml is taken and placed in Eppendorf. Gelatinization occurs after 10 minutes.

This promising result permits to increase concentrations. Two dilutions are prepared, in order to have two different concentrations of  $\beta$ -CD-TEMPO in the AC-PEG formulation (10 mM and 20 mM).

#### Sample E (10 mM)

68 mg of  $\beta$ -CD-TEMPO are mixed in 5 ml of AC PEG formulation and sonicated for 10 minutes at 40 C. Then, 25 mg of agarose are added to the system of  $\beta$ -CD-TEMPO and AC PEG. The system is subjected to electromagnetic stimulation (500 W) for a minute. After irradiation, when the temperature achieves 50 C, 1 ml is taken and placed in Eppendorf. Gelatinization occurs after 10 minutes.

#### Sample F (20 mM)

131 mg of  $\beta$ -CD-TEMPO are mixed in 5 ml of AC PEG formulation and sonicated for 10 minutes at 40 C. Then, 25 mg of agarose are added to the system of  $\beta$ -CD-TEMPO and AC PEG. The system is subjected to electromagnetic stimulation (500 W) for a minute. After irradiation, when the temperature achieves 50 C, 1 ml is taken and placed in Eppendorf. Gelatinization occurs after 10 minutes.

### *PEG-TEMPO FUNCTIONALIZED HYDROGELS*

Two dilutions are prepared, in order to have two different concentrations of **(10)** in water-PBS solution (2 mM and 20 mM). Giving that the product consists on PEG and two unit of TEMPO the molecular weight of the product is above all  $\sim$ 2500 mg/mmol.

10 mg of PEG-TEMPO **(10)** are dissolved in 2 ml of PBS to have a solution 2 mM (sample **G**).

94 mg of PEG-TEMPO **(10)** are dissolved in 2 ml of PBS to have a solution 20 mM (sample **H**).

To 6 ml of the AC-PEG formulation, 30 mg of agarose powder are added and this system is irradiated with microwave radiation at 500 W until boiling, for a time usually between 30 seconds and 1 minute, and electromagnetically heating up to 80 C. At this point the gel is liquid and condensation reactions begins.

When the temperature achieves 50 C , 500  $\mu$ l are taken and placed in Eppendorf where 500  $\mu$ l of the sample **G** (2 mM ) are placed.

The same procedure is done for the sample **H** (20 Mm).

Gelatinization is complete after 20 minutes.

#### *PEG-TEMPO FUNCTIONALIZED HYDROGEL*

Two samples are prepared, in order to have two different concentrations of **(9)** in the AC-PEG formulation (10 mM and 20 mM).

Sample **I** (10 mM)

50 mg of the product are dissolved in 2 ml of formulation. 10 mg of agarose are added.

The system is subjected to electromagnetic stimulation ( 500 W ) . After irradiation , when the temperature reaches 50 C, 1 ml is taken and placed in Eppendorf. Gelatinization is complete after 20 minutes.

Sample **L** (20 mM)

100 mg of the product were dissolved in 2 ml of formulation. 10 mg of agarose were added. The system was subjected to electromagnetic stimulation ( 500 W ) . After irradiation , when the temperature reached 50 C, 1 ml is taken and placed in Eppendorf. Gelatinization is complete after 20 minutes.

#### *PAA-TEMPO FUNCTIONALIZED HYDROGEL*

Two dilutions are prepared, in order to have two different concentrations of **(4)** in PBS solution (2 mM, sample **M** and 20 mM, sample **N**).

We consider 89 mg/mmol as molecular weight of the monomer of PAA-TEMPO.

To 6 ml of the AC-PEG formulation, 30 mg of agarose powder are added and this system is irradiated with microwave radiation at 500 W until boiling, for a time usually between 30 seconds and 1 minute, and electromagnetically heated up to 80 °C. At this point the gel is liquid and condensation reactions begin. When the temperature achieves 50 °C , 500 µl of this gel-solution are placed in an Eppendorf, together with 500 µl of the first dilution of PAA-TEMPO (2 mM ) . The same procedure is repeated with the second dilution (20 Mm). Gelatinization is completed after 20 minutes.

# Biological Tests

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## *MR IMAGING OF HYDROGELS*

Since the last few decades, hydrogels have been extensively studied and used as drug delivery media and tissue repairing materials benefitting from their softness and excellent biocompatibility. Among them, an increasing number of biodegradable and injectable hydrogels has attracted the attention of the researchers because of their flexible utilization methods and reduced surgical trauma. Literature on directly labeling hydrogels with a contrast agent is not so common, even if this field shows many prospective applications. For instance, hydrogels can be employed for carrying drugs and their release *in vivo* could simultaneously be monitored with the help of MRI over a prolonged period.

This chapter shows the results obtained by the Mario Negri Institute for Pharmacological Research in Milan, testing our hydrogels in *in vitro* assays. The aim of this investigation was to demonstrate that this kind of materials can be detected by MRI, being useful contrast agents.

## *SELECTED EXAMPLE*

As a reference, we were inspired by an imaging study of hydrogels chemically labeled with Gadolinium as a contrast agent, reported by Liu, J. and coworkers ( Liu,J.; Wang, K.; Luan, J. et al., *J. Mater. Chem. B*, **2016**, (4), 1343-1353).

These MRI-visible hydrogels are based on chitosan and PEG. Starting from a Schiff's base formation, the hydrogels can be quickly obtained just by mixing the macromolecular chelator of Gadolinium (based on chitosan) and the crosslinker (PEG end capped with aryl aldehyde) at room temperature. The *in vitro* release behavior of the hydrogel was then tested using rhodamine B as a model drug.

The MRI contrast enhancement effect of Gadolinium is show in figure 25.

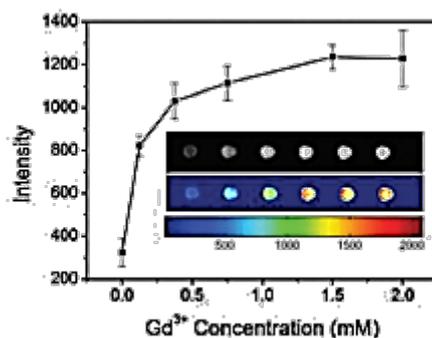


Fig. 25: intensity of MRI visible hydrogel versus the  $Gd^{3+}$  concentration [15]

The curve shows the signal intensity of the MR scanner versus  $Gd^{3+}$  concentration. The signal intensity is linearly dependent on  $Gd^{3+}$  concentration and, when the  $Gd^{3+}$  concentration increases up to 1.5 mM, the signal intensity almost does not increase. Regarding contrast agents, as already mentioned, Gadolinium is known for being highly toxic for human body and it's only its chelate structure the one that minimizes the risk of the release of the metal when used for MRI. Compared to Gadolinium, instead, TEMPOL shows many advantages, such as comparatively high relaxation time, prolonged blood half-life, excellent biocompatibility and safe biodegradation properties. For this reason we chose to synthesize TEMPO grafted hydrogels as potential agents for MRI.

### EXPERIMENTAL DATA

All MRI experiments were performed on a 7 T/30 cm horizontal bore magnet (Bruker-Biospin, Germany) equipped with a 12-cm gradient set capable of supplying up to 400 mT/m. A 72 mm transmit-only birdcage coil was used for excitation and actively decoupled from a 10-mm-diameter saddleshape surface coil, which was used for receiving the signal.

Some of the samples, described in the section of hydrogels synthesis, were tested in the MRI scanner.

The desired linear relationship between the concentration of the contrast agent and the  $(1/T_1)$  value is required from the following formula:

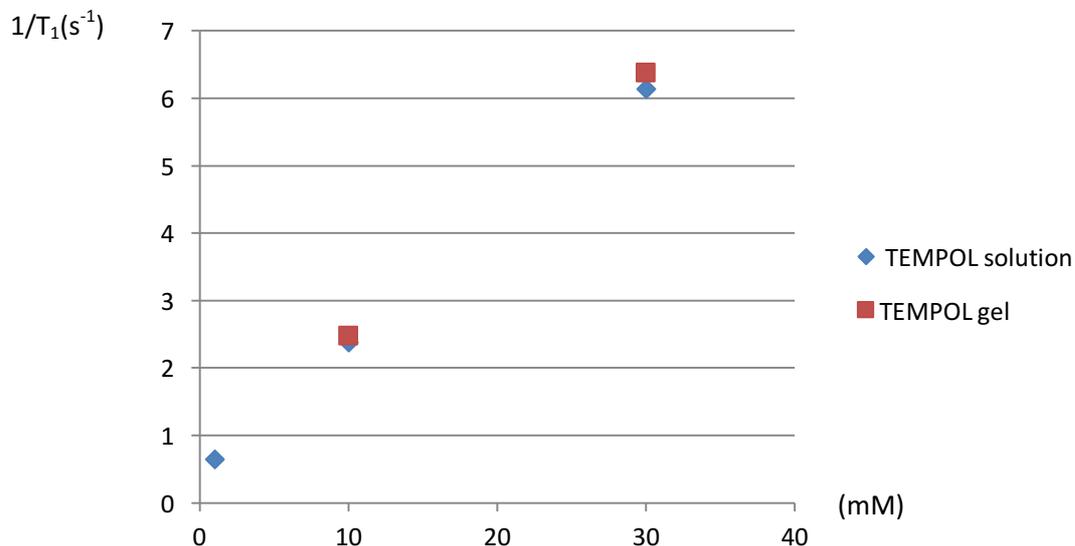
$$1/T_{1,2} = 1/T_{1,2}^0 + r_{1,2} [C]$$

where  $T_{1,2}$  indicates the resulting T1 or T2,  $T_{1,2}^0$  indicates the T1 or T2 of the original tissue,  $r$  is the relaxivity and  $[C]$  is the concentration of the contrast medium.

The relaxivity value of TEMPOL reported in literature is  $0.28 \text{ mM s}^{-1}$  in phosphate buffer at  $25^\circ\text{C}$  and this represent the reference standard.

Graph 1 shows

- different concentrations of TEMPOL in a water-PBS solution versus the relaxation rate ( $1/T_1$ );
- different concentrations of the hydrogel grafted by TEMPOL (sample C and D) versus the relaxation rate ( $1/T_1$ ).

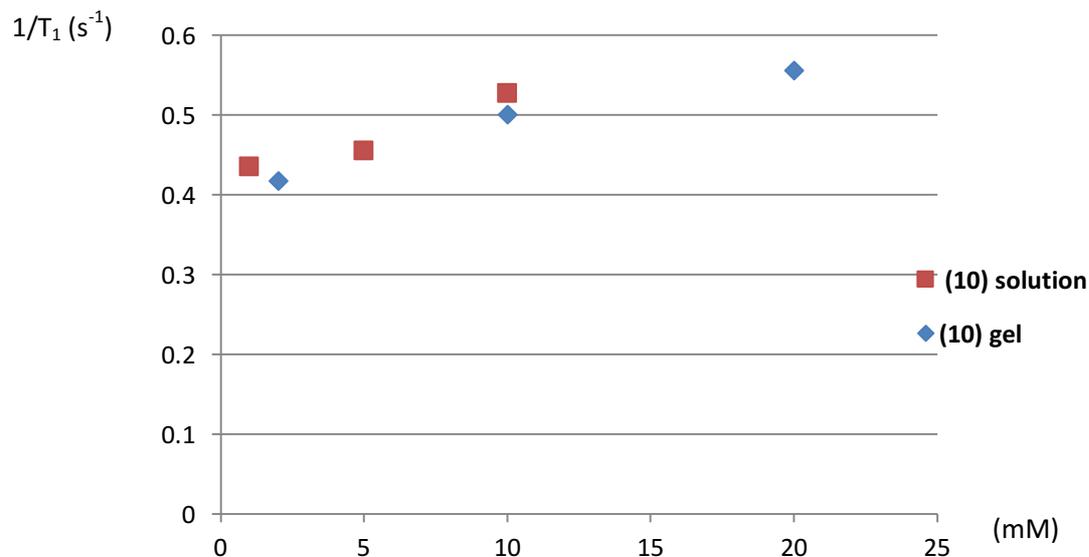


*Graph 1: different concentrations of TEMPOL in a water-PBS solution (red dots) and different concentrations of the hydrogel grafted by TEMPOL (blue dots) versus the relaxation rate ( $1/T_1$ )*

In both cases, a linear relationship between the concentration of the contrast agent and the ( $1/T_1$ ) value is observed. In addition the relaxivity is  $0.19 \text{ mM s}^{-1}$  and it is consistent with the value reported in literature ( $0.28 \text{ mM s}^{-1}$ ) in phosphate buffer at  $25^\circ\text{C}$ .

Graph 2 shows

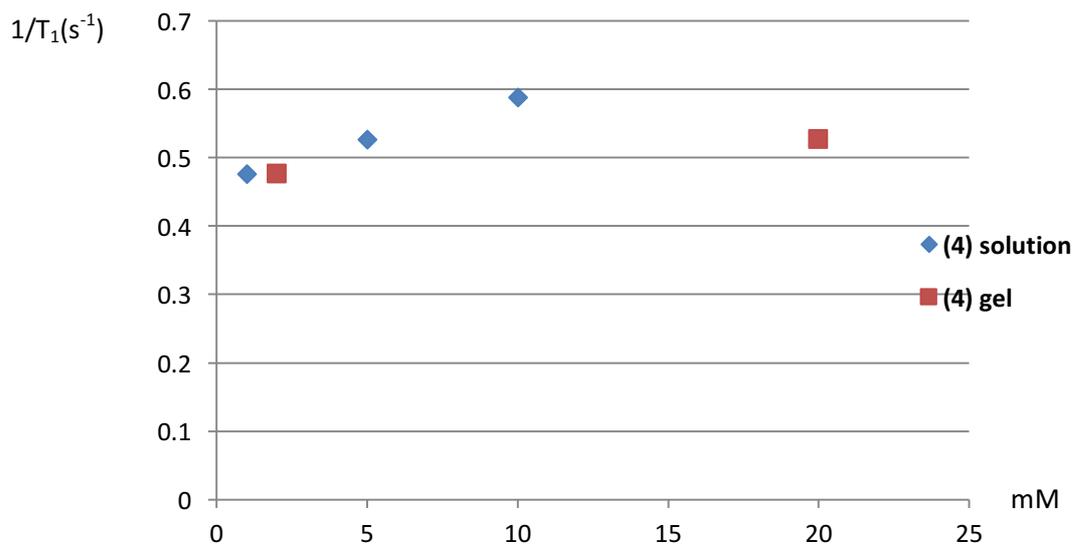
- different concentrations of PEG-TEMPO (**10**) in a water-PBS solution versus the relaxation rate ( $1/T_1$ );
- different concentrations of the PEG-TEMPO (**10**) hydrogel (sample **G** and **H**) versus the relaxation rate ( $1/T_1$ ) .



*Graph 2: different concentrations of PEG-TEMPO (**10**) in a water-PBS solution (red dots) and different concentrations of the PEG-TEMPO (**10**) hydrogel (blue dots) versus the relaxation rate ( $1/T_1$ )*

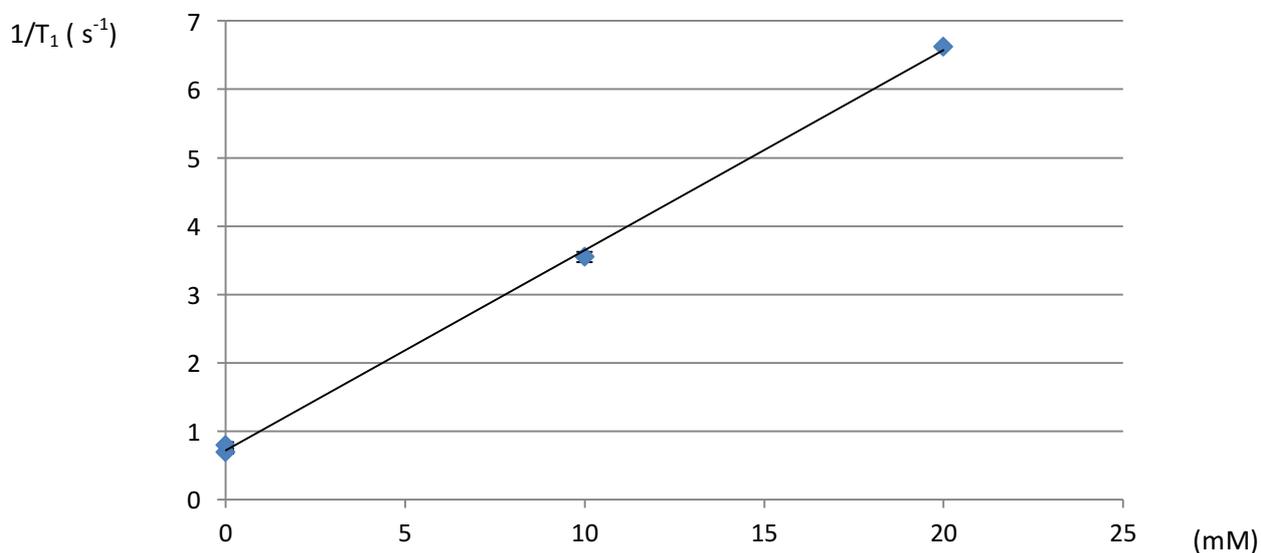
Graph 3 shows

- different concentrations of PAA-TEMPO (**4**) in a water-PBS solution versus the relaxation rate ( $1/T_1$ );
- different concentrations of the PAA-TEMPO (**4**) hydrogel (sample **M** and **N**) versus the relaxation rate ( $1/T_1$ ) .



Graph 3: different concentrations of PAA-TEMPO (4) in a water-PBS solution (blue dots) and different concentrations of the PAA-TEMPO (4) hydrogel (red dots) versus the relaxation rate ( $1/T_1$ )

Graph 4 shows different concentrations of the (9) PEG-TEMPO hydrogel (sample I and L) versus the relaxation rate ( $1/T_1$ ).



Graph 4: different concentrations of the (9) PEG-TEMPO hydrogel versus the relaxation rate ( $1/T_1$ )

A linear relationship between the concentration of the contrast agent and the  $(1/T_1)$  value is observed. In addition the relaxivity is  $0.29 \text{ mM s}^{-1}$  and it is consistent with the value reported in literature ( $0.28 \text{ mM s}^{-1}$ ) in phosphate buffer at  $25^\circ\text{C}$ .

# Conclusions

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In this project the synthesis of new hydrogels as contrast agents media for MRI was investigated. For this purpose, hydrogels were labelled by TEMPO, that is a paramagnetic spin label and, hence, it can be detected by MRI.

The desired functionalized polymers for the hydrogels were based on PAA and PEG. In all cases, we tried two different approaches to functionalize the polymer with TEMPOL: on one hand we took advantage of click chemistry, on the other hand we exploited alternative syntheses.

Hydrogels were prepared starting from AC-PEG formulation and TEMPOL, polymers labelled by TEMPO and  $\beta$ -CD-TEMPO.

It was noticed that the synthesis of these last ones required a sonication cycle, due to the poor solubility of CDs in the formulation.

The evaluation *in vitro* of our materials at Mario Negri Institute for Pharmacological Research in Milan, showed that the value of **(9)** PEG-TEMPO relaxivity is consistent to the one of TEMPOL.

This promising result is going to be evaluated also *in vivo*.

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