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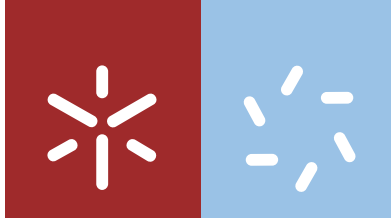
**Multifunctional nanogels containing
magnetic/plasmonic nanoparticles
for therapeutic applications**

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**Multifunctional nanogels containing
magnetic/plasmonic nanoparticles
for therapeutic applications**

Dissertação de Mestrado
Mestrado em Biofísica e Bionanossistemas

Trabalho realizado sob orientação da
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Castanheira Coutinho**
e da
**Professora Doutora Paula Margarida Vidigal
Soares Teixeira Ferreira**

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STATEMENT OF INTEGRITY

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Título: Nanogéis multifuncionais contendo nanopartículas magnéticas/plasmônicas para aplicações terapêuticas

Resumo: Os nanomateriais alteraram profundamente o paradigma tecnológico, industrial e médico, associado com a transição de simples substituintes estruturais para materiais versáteis e multifuncionais. Atualmente, os nanomateriais são procurados como soluções para problemas médicos através da convergência entre a ciência de materiais e a biologia.

Os hidrogéis supramoleculares baseados em péptidos são estruturas fibrilares, entrelaçadas e responsivas a estímulos, auto-organizadas pelo efeito cooperativo de diferentes interações intermoleculares não-covalentes. Atualmente, estão entre os biomateriais mais importantes devido à vasta gama de aplicações, que inclui o transporte de fármacos, engenharia de tecidos, imagiologia *in vivo* e como moldes. A combinação com nanopartículas magnéticas produz magnetogéis, que asseguram o transporte eficiente e a acumulação de fármacos no local terapêutico ou a localização através de um gradiente de campo magnético externo, permitindo aumentar a especificidade de fármacos antitumorais, maioritariamente tóxicos e com efeitos secundários. Além disso, a adição de uma coroa de ouro às nanopartículas magnéticas melhora a biocompatibilidade e possibilita a fototermia, que pode ser explorada como mecanismo para a libertação do fármaco por irradiação.

Neste trabalho, nanopartículas núcleo/corona de ferrite de manganês/ouro e de ferrite de manganês decoradas com ouro foram desenvolvidas e caracterizadas do ponto de vista estrutural, espectroscópico e magnético. Foram sintetizados novos hidrogelantes, tendo sido caracterizados espectroscopicamente e avaliado o seu potencial anti-inflamatório por técnicas computacionais. As nanopartículas foram combinadas com novos hidrogéis supramoleculares baseados em péptidos, formando magnetogéis, estruturalmente caracterizados e testados como transportadores de curcumina (um fármaco com potencialidade antitumoral e neuroprotetiva). Os magnetogéis adequados para transporte foram avaliados como potenciais agentes terapêuticos multimodais, o que incluiu ensaios de interação com modelos de biomembranas, determinação das propriedades magnéticas e reológicas, ensaios de libertação de fármacos e avaliação das capacidades de fototermia. Os resultados são promissores para o uso dos novos magnetogéis na terapia multimodal do cancro, devido à elevada capacidade de carga, à sinergia entre o transporte de fármacos e a localização magnética, e à hipertermia magnética e fototérmica, visionando-se um aumento da eficiência terapêutica no tratamento do cancro.

Palavras chave: Terapia do cancro; Hipertermia; Nanopartículas Magnéticas, Magnetogéis; Plasmónica

Title: Multifunctional nanogels containing magnetic/plasmonic nanoparticles for therapeutic applications

Abstract: Nanomaterials have propelled deep changes in the technological, industrial and medical paradigm, which was accompanied by its translation from simple structural substituents to versatile multifunctional materials. Currently, nanomaterials are sought as solutions to modern medical problems through the convergence of materials science with biology.

Supramolecular peptide-based hydrogels are stimulus-responsive self-assembled intertwined fibrillar structures achieved through the cooperative effect of different non-covalent intermolecular interactions. These are among the most important classes of biomaterials owing to the wide range of applications, which include, among others, drug delivery, tissue engineering, *in vivo* imaging and template materials. The combination with magnetic nanoparticles affords magnetogels, which ensure efficient transport and accumulation of drugs in the therapeutic sites or localization by using an external magnetic field gradient, thus enhancing the specificity of anticancer drugs, most of them toxic and with systemic side effects. Moreover, the addition of a gold shell to the magnetic nanoparticles improves biocompatibility and allows photothermia therapy, which can be used as a photo-triggered release mechanism of the encapsulated drug.

In this work, core/shell manganese ferrite/gold and gold-decorated manganese ferrite nanoparticles were developed, and their structural, spectroscopic and magnetic properties were characterized. The nanoparticles were combined with two new self-assembled peptide-based hydrogels. The hydrogelators were spectroscopically characterized and evaluated as anti-inflammatory agents by computational techniques. The hydrogels and magnetogels were structurally characterized and tested as nanocarriers for curcumin (an anticancer and neuroprotective potential drug). The systems that were suitable carriers were also screened as potential multimodal therapeutic agents. Screening assays included the interaction with biomembrane models, determination of magnetic and rheological properties, drug release assays and photothermia ability. The obtained results are promising for the use of the new magnetogels in multimodal cancer therapy, owing to the high-loading capacity, synergistic magnetically-guided drug delivery, magnetic hyperthermia and photothermia, which are expected to improve therapeutic efficiency in cancer therapy.

Keywords: Cancer therapy, Hyperthermia, Magnetic nanoparticles, Magnetogels, Plasmonics

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Abbreviation list

LMWH	Low-molecular-weight hydrogelator
DNA	Deoxyribonucleic acid
Fmoc	Fluorenylmethoxycarbonyl
FF	Diphenylalanine
RGD	Arginylglycylaspartic acid
SPR	Surface plasmon resonance
PTT	Photothermia therapy
ROS	Reactive oxygen species
AMF	Alternating magnetic field
AC	Alternating current
MRI	Magnetic resonance imaging
EPR	Enhanced permeating and retention
MAR	Motional averaging regime
VLR	Visit limited regime
SDR	Static dephasing regime
HOMO	Highest occupied molecular orbital
LUMO	Lowest unoccupied molecular orbital
FRET	Förster resonance energy transfer
FTIR	Fourier-transform infrared
ATR	Attenuated total reflectance
NMR	Nuclear magnetic resonance
XRD	X-ray diffraction
DFT	Density functional theory
Npx	Naproxen
Met	Methionine
Δ Phe	Dehydrophenylalanine
Lys	Lysine
COX-2	Cyclooxygenase-2
COX-1	Cyclooxygenase-1
DMSO	Dimethyl sulfoxide
CDI	1,1'-Carbonyldiimidazole
MPA	3-Mercaptopropionic acid
DMF	Dimethylformamide
CD	Circular dichroism

TEM	Transmission electron microscopy
SQUID	Superconducting quantum interference device
DCC	<i>N,N'</i> -dicyclohexylcarbodiimide
HOBt	1-hydroxybenzotriazole
Boc ₂ O	Di-tert-butyl dicarbonate
TFA	Trifluoroacetic acid
DMAP	4-dimethylaminopyridine
TMG	<i>N,N,N',N'</i> -tetramethylguadine
CGC	Critical gelation concentration
MEP	Molecular electrostatic potential
RMSD	Root mean square deviation
Tyr	Tyrosine
Arg	Arginine
Leu	Leucine
Val	Valine
Ala	Alanine
Trp	Tryptophan
Ser	Serine
Pro	Proline
Glu	Glutamate

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Chapter 1 - Introduction

1.1. Nanomedicine

The advancements on control of the matter properties at nanoscale, i.e. at dimensions between 1 and 100 nanometres, have propelled revolutions at the technological, industrial and medical level [1,2]. Particularly, the convergence of materials science with biology accompanied by the recent synthesis, manipulation and visualization methods of materials at the nanoscale has contributed to the consolidation of a new area of nanomedicine, which seeks to solve modern medical problems through nanotechnology.

The progression of nanomaterials towards multifunctionality has opened new paths on the development of strategies towards drug delivery, cell differentiation, transmission of information and advances towards better contrast agents [3-6], apart from stimulating a greener economy through the development of new energetic, electronic and depollution technologies [7].

Cancer keeps rising every year, with lung, colorectal, breast and prostate exhibiting the highest incidence ratio [8-10]. Such high ratio is becoming an increasing problem in low-income countries but also in high-income ones [8,9]. Hereby, besides the individual genetical predisposition to develop cancer, its high incidence is not only associated with the economic transition induced changes in social, economic and cultural relations, but also the adoption of unhealthy lifestyles of the high-income countries, such as tobacco, physical inactivity and excess body weight [9-13]. Therefore, the promotion of healthy lifestyles, tobacco control, vaccination, early detection and improved treatments are highly required [9,10,13].

Efforts have been made on improving cancer survival rates through conventional chemotherapy, but such has only been practically verified in high-income countries and particularly in patients with early stages of the disease [13,14]. However, patient's quality of life, target specificity, therapeutic effectiveness and cost remain as a major problem to solve, not only to reduce the adverse effects on healthy cells, but also to extend better and affordable treatments to low-income countries.

Therefore, new nanosystems with high performance in therapeutics are required, through the improvement of target specificity and addition of further features, such as enhanced magnetic resonance imageology, biosensing and hyperthermia.

1.2. Hydrogels

Since the pioneering work of Wichterle and Lim, who developed the first synthetic hydrogel [15], the field of hydrogels has been an area of intensive research in materials science, which has found use in a wide range of applications and positively stimulates a direct competition with other commercialized products.

Hydrogels are elastic three-dimensional networks made of natural/synthetic water-soluble polymers [16-18]. The leading interactions involved in the network cross-linking or entanglement promote their water insolubility accompanied by the retention of a huge portion of solvent, that ultimately results in gel formation [18,19]. Hereby, the gelation process is characterized by a network expansion due to the capillary, osmotic and solvation forces that are counterbalanced by forces that resist expansion and keep network integrity [20]. The equilibrium between these opposing forces and its magnitude will determine the hydrogel intrinsic properties, such as the internal transport, diffusion characteristics and mechanical strength, which are also governed by the network morphology and chemical nature of the network chains [20].

The volume transition associated with gelation can be controlled by a wide variety of physical and chemical stimuli, such as temperature, pH, sound, light, pressure, ionic force, electric or magnetic field [20]. Moreover, as the three-dimensional structure can be driven by physical or chemical interactions, the swelling and mechanical properties are differently affected according to the gelation mechanism. In chemical hydrogels, cross-linking occurs through covalent bonds, while, in physical hydrogels, is due to non-covalent interactions [20].

Therefore, the core strategy is centred in taking advantage of the vast diversity of usable polymers, producible physical forms and stimuli to activate hydrogel formation to conceive the required physical and chemical properties [16,17,21,22]. This versatility is highly relevant in the development and tailoring of nanosystems, as it allows precise control over drug diffusion and range of drugs capable of being incorporated through changes of the network structure porosity, the hydrogelator affinity to the aqueous environment and the density of reticulate agents [16,23]. These properties span the macroscopic scale characteristics of hydrogels, i.e. the size and porous structure, as they influence the administration route and macroscopic physical properties (figure 1.1), such as the deformability [24]. At the mesh scale, the open spaces size (mesh size) affects the temporal and stimulus-responsive drug diffusion inside the hydrogel network, while at the molecular and atomistic scale, the network chains might have physical or chemical binding sites that interact with the loaded drug (covalent conjugation, electrostatic interaction or hydrophobic associations) and can affect the sustained or on-demand drug release [24].

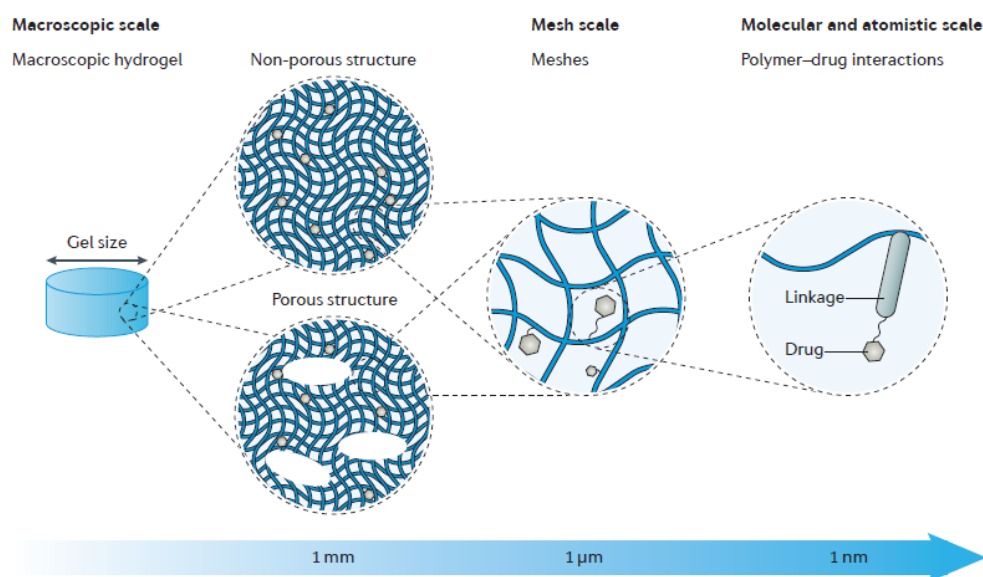


Figure 1.1. At the macroscopic scale, hydrogel can be non-porous or contain pores that are 10-500 μ m, while the mesh size is generally tuneable in the range 5-100 nm. At the molecular or atomistic scale, the loaded drug can interact with the network chains (e.g. covalent linkage). Adapted from [24].

Recently, self-assembled (physical) hydrogels have acquired a remarkable plethora of applications either in tissue engineering, *in vivo* imaging or drug delivery, owing to their high portion of water and physicochemical similarity to the cellular matrix, both in composition and mechanical properties, but also due to its cost-effective, versatile, facile, stable and robust structures [25–34]. Considering that the gelation is achieved through the cooperative effect of different non-covalent intermolecular interactions, such as hydrogen bonding, van der Waals, electrostatic, hydrophobic and aromatic π - π interactions [17,21,25–28], the fundamental aspect from a physicochemical point of view is that the molecule must be amphiphilic, i.e. needs a hydrophilic region that interacts with water and a hydrophobic region that aggregates into a core [34]. A direct consequence of the wide variety of interactions involved in the gelation is that the design, preparation and manipulation of the hydrogelators must be rationally conceived, since it will be affected by several factors, such as pH value, kinetics of pH drop, temperature, ionic force and structure amphiphilicity [17,21,26–33]. Nevertheless, such aspects become an advantage, as the design can be rationalized from a library of compounds with the potentiality to be further modified with responsiveness to enzymatic, hydrolytic or environmental pathways.

1.2.1. Supramolecular hydrogels

Supramolecular hydrogels are a novel class of physical hydrogels that comprise low-molecular weight hydrogelators such as oligopeptides, amphiphilic peptides and peptides conjugated to an aromatic moiety, or polymers that self-assemble into hierarchically-organized structures [26,35,36]. The reversible and spontaneous self-assembly of the initially randomly-oriented hydrogelators produce stable and well-defined structures due to localized inter- and intramolecular interactions, which will also affect the interaction of the resulting supramolecular architecture with other molecules, cells and tissues [36-38]. Nevertheless, self-assembly into a hydrogel requires a trigger that provides the required free energy either by inducing a negative enthalpy and/or a positive entropy variation, which can be attained through physical (e.g. temperature, ultrasounds or ionic force) or chemical (e.g. pH or photochemical reaction) methods [38]. Moreover, considering the forces that are involved in the self-assembly and phase transition process, the conformation and stability of the supramolecular architecture will not only be affected by the molecular nature and final solution conditions, but also by the route and kinetics to reach those conditions [39].

The most common triggering method is heating the hydrogelator aqueous solution and cooling the supersaturated isotropic solution until room temperature [29,40]. After the stimulus, the gelation process is induced, which will result: (I) in the formation of a crystal, due to high ordered aggregates; (II) random aggregation into a precipitate; (III) a metastable state (hydrogel) as a product of optimum intermolecular forces during a homogeneous self-assembly [29]. The resulting reversible supramolecular intertwined fibrillar structure is heterogeneous, with sizes ranging from nano- to micrometres, as a result of the hierarchical organization [40], where the solvent pocket microdomains can retain nanomaterials such as nanoparticles and nanotubes [29].

The supramolecular low-molecular-weight hydrogelators (LMWH) have various advantages over the polymeric, such as low bioaccumulation owing to the lower hydrogelator concentration required to attain a hydrogel, which can be effectively removed *via* the renal system; structural versatility (fibres, nanoparticles, nanotubes, micelles); easy chemical functionalization; and manufacture at low cost [33,36]. Moreover, it is possible to develop hydrogels using different moieties, such as cholesterol derivatives, surfactants, carbohydrates, amides, long-chain alkanes and amino acid derivatives [33].

The amino acid-based LMWH are particularly adequate for applications in nanomedicine and bionanotechnology, owing to the tailoring without dangerous chemicals, the easy metabolization of amino acids, non-immunogenicity, non-thrombogenicity, optimum biodegradability and applicability in localized

therapies [36]. Moreover, small peptides do not suffer from the structural complexity and delicacy of therapeutic proteins [41], making it easier to establish structure-function relations. The use of polar amino acids provides hydrogen bonds; aromatic amino acids will favour the π - π stacking of the hydrogelators; non-polar amino acids induce the hydrophobic collapse; and the acidic or basic amino acids add electrostatic interactions [41]. Through modulation of the peptide blocks it is possible to control the degradation and diffusion properties of hydrogels and even adapt it to encapsulate biological entities, such as enzymes [41].

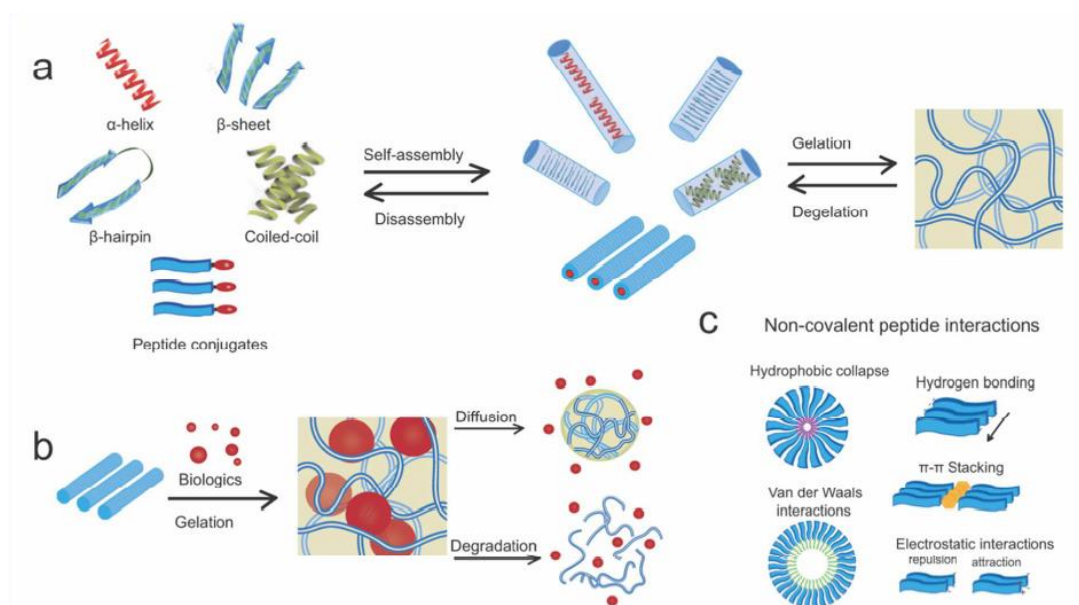


Figure 1.2. Schematic representation of (A) the peptide-based supramolecular hydrogelators self-assembly in hydrogels, (B) the encapsulation and release of biological entities, and (C) the non-covalent interaction associated with hydrogelation. Adapted from [41].

Currently, there is a wide variety of hydrogelator design strategies, such as ionic self-complementary peptides, β -hairpin peptides, multidomain peptides, ultrashort peptides (≤ 7 amino acids), conjugated peptides and peptide amphiphiles [42]. However, conjugated peptides have been more appealing for biomedical applications owing to the fast synthesis and easy modulation. The general structure is an aryl motif such as ferrocenyl, fluorenyl or naphthyl conjugated to a di- or tripeptide block, where the former provides increased π - π interactions, stability and enhanced hydrogelation owing to its tendency to form a core of stacked aromatic groups, beside the steric optimization from the linker [41]. A depletion of aromatic moieties in the peptide backbone might induce other structures such as spheres or discrete tubular morphologies [39]. The addition of aromatic moieties favours self-assembly yield, higher thermal stability, elasticity and faster self-assembly [39]. Moreover, the use of a dehydroamino acid, such as dehydrophenylalanine adds conformational constraint in the peptide backbone that favours hydrogelation and provides increased resistance to enzymatic degradation [43].

1.2.2. Applications of hydrogels

The high-water content, flexibility, biocompatibility and versatility have contributed to the use of hydrogels in a wide range of applications, such as tissue engineering, biosensors, hygienic products, drug delivery, contact lenses, cell therapy, 3D cell culture, agriculture and horticulture [16,20,25,44].

The deformability favours the hydrogel conformation to a surface, while the muco- and bioadhesive properties strengthen its attachment, which can be further reinforced if cationic groups are present. These groups will also interact with DNA negative charges and can be explored on the development of non-viral vectors or bioinks for 3D cell culture and biofabrication [16,20,45,46].

Supramolecular hydrogels have shown promising results in tridimensional cell culture, for example Zhou *et al.* demonstrated that the co-assembled Fmoc-FF (Fluorenylmethoxycarbonyl-diphenylalanine) and Fmoc-RGD (arginylglycylaspartic acid) hydrogel could work as a scaffold for 3D culture of human dermal fibroblasts [47]. Fibroblasts deposited dense and organized extracellular matrix networks, including fibronectin and collagen in a controlled manner within 14 days [47].

The development of bio-inspired catalysts has become a hotspot for hydrogels as the next-generation enzymatic mimics, where a recent advance was the thermoreversible tripeptide-based histidylphenylphenylalanine hydrogel developed by Garcia *et al.* that displayed esterase-like activity [48].

Hydrogels' controllable porous structure can be adapted to favour encapsulation of drugs in the matrix, besides modulating its release through changes that affect its diffusion coefficient, which guarantees a high drug concentration in the target site, for a long period of time [25]. Considering the abovementioned hydrogel properties, the applicability in different forms such as rectal [49], nasal [18], topic and intravenous injection can be optimized by adapting the mechanical and shape properties that best fit the required parameters to maximize the overall efficacy and patient compliance (figure 1.3). Macroscopic hydrogels are adequate for surgical implants, transepithelial and transdermal drug delivery, which is required when the target site is located deep within the tissue or when the biological barriers display low permeability to the drug to be administrated [24]. However, owing to the associated risks of surgical implantation, the alternative administration route of macroscopic hydrogels has been the *in situ*-gelation, shear-thinning hydrogels (flow when a shear stress is applied) or shape-memory hydrogels (can collapse and recover the initial structure) [24]. A minimal invasive solution are the microgels and nanogels, which, due to the small size, are needle-injectable, lead to facile natural clearance and enhance penetration through the physiological barriers, thus providing alternative routes such as oral, pulmonary, intrabony and systemic drug administration [24].

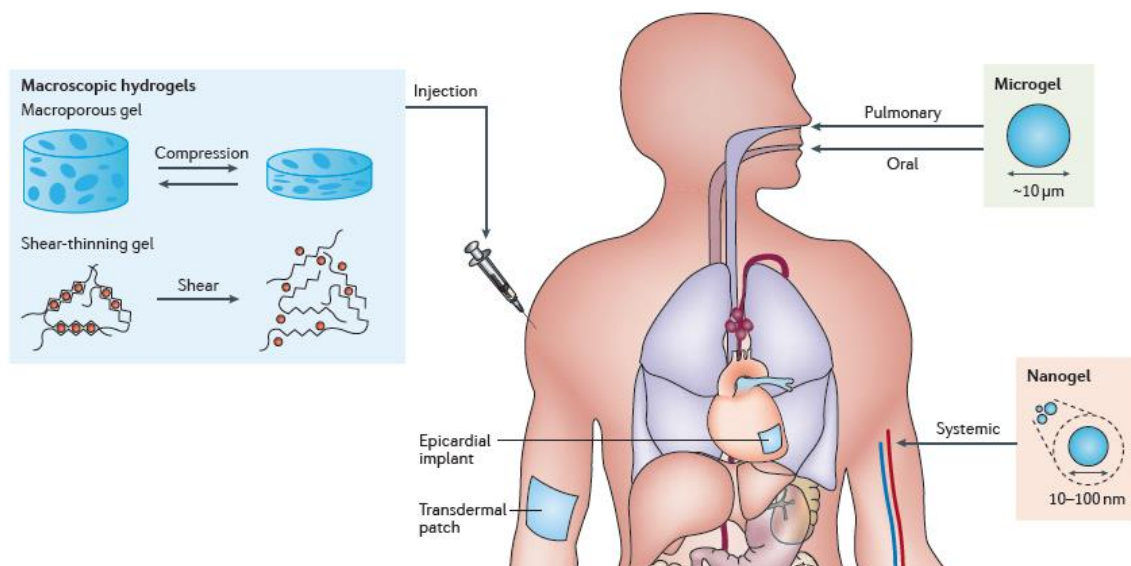


Figure 1.3. Schematic representation of the possible and optimum administration routes for each hydrogel shape. Adapted from [24].

Currently, the development of hydrogels focuses on enhancing drug delivery efficiency by improving control over drug release rate and amplifying the range of compounds possible of being encapsulated [16,23]. The former is performed through modulation of the interaction between the hydrogel matrix and the drug of interest, while the latter requires the modification of the drug release diffusion barrier that is affected by the mesh size [16]. Release predominantly occurs through diffusion and is independent on the mesh size when the drug size is smaller than the mesh size, while steric hindrance becomes prominent for mesh sizes close to the drug size, which slows diffusion owing to frictional drag on the diffusing drug [24]. If the drug size is larger than the mesh size it becomes immobilized and release will occur either by mesh degradation, deformation or swelling [24].

Although hydrogels possess a hydrophilic matrix that might difficult the encapsulation of hydrophobic compounds, the hydrogel matrix can be tailored with hydrophobic domains [23]. Therefore, as hydrogels show chemical or physical stimuli responsiveness, reduce chemotherapeutic drug cytotoxicity and increase drug concentration on the therapeutic target, it is a highly advantageous and controllable nanosystem suitable for biomedical applications.

1.3. Nanoparticles properties

Nanoparticles are materials with sizes below 100 nm at least in one dimension, where the quantum phenomena have a great impact on the materials properties such as the physical, optical, magnetic, electronic and surface properties, which are common to all materials independently of the chemical composition [50,51].

The electronic and chemical properties are determined by the distribution of electrons in the valence and conductance bands. In metals the two bands overlap, but in semiconductors and isolators an energy gap separates the bands, where the excitation of an electron produces a positively charged hole in the valence band [52]. The excited electron becomes delocalized, i.e. moves freely within the atomic lattice, which is an important phenomenon to the conduction process. Nevertheless, the size reduction confines the random movement of electrons to discrete levels, leading to larger energy gaps and, eventually, small enough metal nanoparticles can behave as semiconductors [52]. Consequently, the optical band, i.e. the energy required to activate the electron-hole complex, but not to release the electron to the conductance band, is also increased, which is characterized by a blue shift of the absorbance spectrum [50].

The increased surface area promotes higher percentage of surface atoms, which is accompanied by a reduction of the melting and evaporation temperatures, and the cohesive energy [50]. Moreover, the higher number of uncompensated spins enhances the surface free energy, which can be reduced by coating with another material. Parameters such as the low coordination number, large free energy, anisotropy, bond defects and surface tension determine the properties of the nanoparticle. Therefore, a coating might be used to modulate nanoparticle interaction with tissues, as the reduced size and large surface favour the interaction with bulk materials surface, through non-covalent interactions, non-covalent electron sharing and fitting in the grooves [50,53,54].

On the other hand, the incorporation of nanoparticles into polymeric materials induces tensile forces changes, owing to the interactions between the nanoparticles and polymeric networks. Hereby, the modulation of the nanoparticles size and surface properties promotes the manipulation of the nanocarriers physicochemical properties, such as pharmacokinetics, distribution, accumulation, toxicity and adaptation of nanoparticles to the necessities [51].

1.3.1. Magnetic nanoparticles

The complex development of self-assembly techniques to attain highly organized structures and synthesis of magnetic nanoparticles was originally pioneered by nature, which prompted the interest for its in-laboratory reproduction to adapt the nanostructures to different technological applications [55]. The advances on synthesis have eased the manipulation and manufacture of nanoparticles with the desired properties, which in combination with the inherent interdisciplinarity have promoted the use of nanoparticles in various technologies, such as ferrofluids, catalytic, magnetic and electronic systems [56]. Nevertheless, most of the investment and research focus on the biomedical applications, which has contributed on the optimization of contrast agents for magnetic resonance imaging, the use of hyperthermia as an adjuvant cancer therapy, the conceptualization of nanoparticles as nanocarriers and the possibility of manipulating cell membranes [56,57].

The main phenomenon associated with its wide range of applications is the superparamagnetism, which occurs due to the finite size and surface effects that dominate the magnetic behaviour [58]. Essentially, superparamagnetism is the absence of remanence magnetization after the application of an external magnetic field [59], i.e. magnetization only occurs in the presence of an external magnetic field. This guarantees colloidal stability and averts aggregation after removing the magnetic field, thus promoting the introduction of magnetic nanoparticles in biomedical applications. Moreover, the nanoparticles magnetization is characteristically homogeneous and of high magnetic susceptibility above a certain temperature (blocking temperature) [58].

Generally, magnetic nanoparticles are made up of metallic elements, such as iron, nickel, manganese, cobalt and its oxides [59], with or without a shell. Ferrites and its oxides, such as magnetite and its oxidized form, maghemite, are the most common used nanoparticles in biomedical applications owing to the good colloidal stability, no remanence magnetization, and a surface possible of being easily modified with an organic or inorganic shell [58,60]. The addition of a silica shell further improves its chemical and colloidal stability, size distribution, manipulation over magnetic moment and surface covalent functionalization [58,60]. The nickel and cobalt metallic nanoparticles are highly toxic and prone to oxidation, though have higher magnetic moments than ferrites. They can also be obtained with smaller sizes and can be adapted to biomedical applications through addition of an inorganic shell that provides stability to acidic and basic conditions and conserve the high magnetization [60].

Nowadays, the biocompatibility, chemical stability and fine synthesis of iron oxide nanoparticles have contributed for its wide application in hyperthermia, imageology and radiotherapy [61].

1.3.2. Superparamagnetism

Every spinning electric-charged particle with mass, such as electrons, protons or ions, create a magnetic dipole (magneton), which in the electron is a result of the orbital and spin magnetic dipole that can vectorially sum or cancel each other [58].

Magnetic materials are classified according to the response to an externally applied magnetic field into five main types: diamagnetic, paramagnetic, ferromagnetic, ferrimagnetic and anti-ferrimagnetic [58,62]. Diamagnetic materials (Cu, Ag and Au) have no unpaired electrons, which results in a zero net magnetic moment and weakly repels an externally applied magnetic field, thus having a negative magnetic susceptibility [58]. Paramagnetic materials (Gd, Mg and Li) have a magnetic moment owing to the unpaired electrons, which align with an externally applied magnetic field and produces a weak net magnetic moment with a slightly positive susceptibility [62]. Moreover, these materials have no remanence magnetization due to a lack of long-range ordering of the magnetic moments [58]. Ferromagnetic materials (Fe, Ni and Co) have parallelly aligned magnetic moments that are organized in domains, which is associated with the long-range ordering exchange interactions of the magnetic moments. In the presence of an external magnetic field the domains align, and the direct coupling interactions favour an enhancement of the flux density [58], thus attaining a large magnetic moment (positive magnetic susceptibility). Antiferromagnetic materials have antiparallelly coupled magnetic moments of equal magnitude, while ferrimagnetic materials also possess two different atoms in two different lattice positions with opposite magnetic moment, but different magnitude that produces a net spontaneous magnetic moment [62]. However, the collinearity is only kept until a Néel temperature, i.e. the temperature above which the magnetic moment randomly fluctuates, and long-range order is lost, which leads the material to exhibit a paramagnetic behaviour [58,63].

In bulk magnetic materials, the magnetic domains (Weiss domains) are divided by Bloch walls, as a result of several energy terms: the exchange energy, that tries to align the magnetic moments, magnetocrystalline anisotropy, which orients the magnetic moments in a preferential orientation, and the magnetostatic energy that seeks to eliminate the magnetization of the material [63].

An externally applied magnetic moment orients the Weiss domains magnetic dipoles accompanied by the disappearance of the Bloch walls until a saturation magnetization (M_s) is attained [64]. Upon removal of the magnetic field, a residual magnetisation (M_r) is preserved, thus the nanoparticles show a M-H hysteresis loop, i.e. it requires a coercive force to bring the magnetization down to zero. A magnetization

cycle has an associated energy lost by heat dissipation (hysteresis losses) that can be measured from the hysteresis cycle integration (figure 1.4) [64].

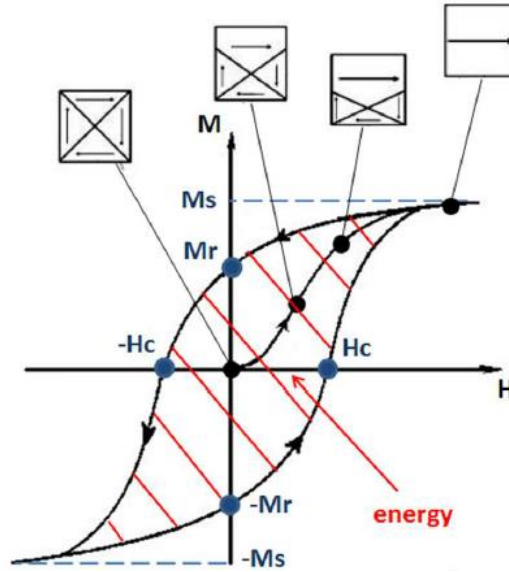


Figure 1.4. Schematic representation of the ferromagnetic materials hysteresis loop, where the squares represent the Bloch walls motion induced by an external applied magnetic field. Legend: H_c is the coercivity; M_s is the saturation magnetization; M_r is the residual magnetization. Reprinted from [57].

Nevertheless, as the magnetostatic energy is proportional to the particle volume, while the energy required to form a Bloch wall is proportional to the wall-domain interface area, there is a critical radius (r) below which it is more energetically favourable to support the magnetostatic energy cost than forming a Bloch wall, leading to the formation of single-domain nanoparticles. The critical radius can be estimated, for a spherical particle, from the equation [64]:

$$R = \frac{72\sqrt{AK}}{\mu_0 M_s^2} \quad (1.1)$$

where A is the exchange resistance constant, which is related to the critical temperature to keep magnetic order, K is the magnetic anisotropy, M_s is the saturation magnetization and μ_0 is the magnetic permeability in the vacuum.

Unlike bulk magnetic materials, single-domain nanoparticles are uniformly magnetized, and magnetization occurs by spin reversal (or Néel relaxation) or particle motion (Brown relaxation, which causes friction with the medium) instead of the Bloch walls motion. Such effect, combined with the nanoparticle shape anisotropy, produces a higher coercivity than in the multi-domain magnetic materials [62].

The magnetic behaviour of an uniaxial single-domain nanoparticle can be approximated by the Stoner-Wohlfarth model and the associated energy to rotate along an easy axis can be expressed by the anisotropic energy (E_a) and Zeeman energy (E_Z) (figure 1.5) [64,65]:

$$E = E_a + E_Z = K_T V \sin^2 \theta - H V M_S \cos(\theta - \Phi) \quad (1.2)$$

where, in the first term (anisotropy energy), K_T corresponds to the total anisotropy constant, V to the particle volume, θ to the angle between the magnetization direction of the particle and the easy magnetization axis (0 or π radians). In the second term, H is the applied magnetic field and Φ is the angle between the easy axis and the applied magnetic field.

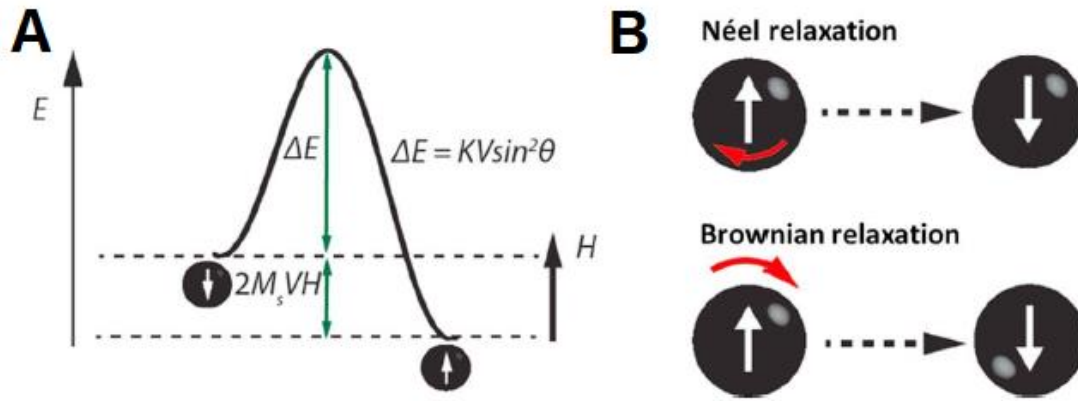


Figure 1.5. Schematic representation of (A) the energy barrier required to change the orientation of a magnetic nanoparticle under an applied magnetic field (H) and (B) the relaxation processes involved on the reorientation of the nanoparticle's magnetic moment. Adapted from [65].

As the volume decreases (at $H = 0$), the required energy to invert the magnetic moment along the easy axis becomes comparable or smaller than the thermal energy ($E_T = k_B T$, where k_B is the Boltzmann constant and T is the temperature). The temperature at which nanoparticles attain the superparamagnetism state is the blocking temperature, defined by the equation [65]:

$$T_B = \frac{K_T V}{k_B \ln(\frac{\tau_m}{\tau_0})} \quad (1.3)$$

where τ_m is the measurement time and τ_0 is the attempt time (length of time characteristic of the material). Above the blocking temperature, the particle is said to be superparamagnetic as it rotates freely in the absence of an external magnetic field, showing no coercivity and hysteresis [64].

1.3.3. Plasmonic nanoparticles

Since the traditional Chinese medicinal use of gold salts for the treatment of rheumatoid arthritis, gold has been one of the most interesting materials for biomedical applications [66-69]. The easy surface functionalization, possible synthesisable shapes (figure 1.6), biocompatibility, low toxicity and the surface plasmon resonance (SPR) phenomenon, contribute to the use of gold nanoparticles as a highly advantageous material in several biomedical applications [66-68].



Figure 1.6. Examples gold-base nanoparticle shapes. Reprinted from [66].

The gold nanoparticles synthesis has undergone various changes in order to improve control over size, shape and functionality. A common method is the reduction of hydrogen tetrachloroaurate (III) (HAuCl_4) with citrate in boiling water, which has the dual role of stabilizing and reducing agent [68]. Although control over size is possible through manipulation of gold-to-citrate ratio, surfactants are needed to prevent the formation of irreversible aggregates during functionalization with thiolate ligands.

A second common method is based on a two phases synthesis that makes use of tetraoctylammonium bromide (TOAB) as a phase transfer reagent and sodium borohydride as the reducing agent. This method offers low polydispersity according to the synthesis parameters, such as gold-to-reducing agent ratio, temperature, reagents concentration, and stabilizing polymers [68]. The strong S-Au interaction and van der Waals attraction between neighbouring ligands favours the stabilization of nanoparticles obtained by this route [68].

The surface plasmon resonance (SPR) is the phenomenon associated with the interest of using gold nanoparticles in cancer therapy, which consists on the collective motion of the conduction electrons in the crystal lattice induced by an externally applied electric field. A depolarizing electric field is formed upon oscillation, which acts as a restoring force (figure 1.7), where the interaction between the electrons and the environment/vicinity leads to a damped oscillator-like behaviour [68]. Nevertheless, such behaviour requires the size of the nanoparticle to be smaller than the photon wavelength, so that plasmon oscillation is distributed along the whole nanoparticle volume, which is hindered for particles smaller than 2 nm, owing to the quantization energy of the electronic levels becoming high enough, i.e. the nanoparticle starts behaving as a semiconductor.

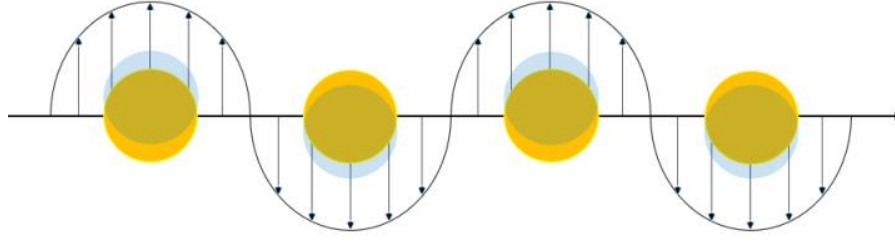


Figure 1.7. Schematic representation of the conduction electrons collective motion induced by the electromagnetic radiation.

The extinction coefficient (σ_{Ext}) can be approximated for a quasistatic regime (nanoparticle smaller than the wavelength) according to the equation [70]:

$$\sigma_{Ext} = \frac{18\pi[\varepsilon_m(\lambda)]^{3/2}}{\lambda} V \frac{Im[\varepsilon(\lambda)]}{[Re[\varepsilon(\lambda)] + 2\varepsilon_m(\lambda)]^2 + Im[\varepsilon(\lambda)]^2} \quad (1.4)$$

where ε_m is the dielectric constant of the non-absorbing medium, λ is the wavelength, $\varepsilon(\lambda)$ is the wavelength/frequency dependent complex dielectric function of the nanoparticle, and V is the nanoparticle volume. From the above equation, resonance will occur when $Re[\varepsilon(\lambda)] \sim -\chi\varepsilon_m(\lambda)$ (χ is a geometrical factor equal to 2 for a sphere). Hereby, the surface plasmon resonance depends on both particle size and environment dielectric constant, which means that the gold nanoparticles can be used as sensors considering that the bonding of molecules or their proximity to the nanoparticle surface will modify the medium dielectric constant and, thus, the surface plasmon resonance frequency [68]. Moreover, the surface plasmon band is also sensible to the type of ligand, core charge, temperature, shape and aggregation [68]. The aggregation is a commonly used phenomenon for sensors, as it induces a bathochromic shift and bandwidth widening of the SPR band and a change of the solution from red to blue, due to the interparticle plasmonic coupling [68]. Another phenomenon is the fluorescence quenching due to Förster resonance energy transfer (FRET) or modulated photoinduced electron transfer, which led to the application of gold nanoparticles in different technological areas, ranging from biosensors to the monitorization of biological cells [66,68,70-72].

The fast phase loss of the coherently excited electrons allows conversion of the absorbed light into heat through nonradiative processes. The nanoparticle local heating (q_{NP}) is dependent on the plasmonic high absorption cross-section (typically, for sizes below 20 nm) according to the equation [70,71,73]:

$$q_{NP} = \sigma_{Abs}(\lambda)I(\lambda) = \frac{1}{2}\omega\varepsilon_m \int Im[\varepsilon(\lambda)]E_{NP}^2(\lambda)dV \quad (1.5)$$

where $I(\lambda)$ is the incident light intensity, ω is the angular frequency and E_{NP} is the electric field amplitude inside the nanoparticle. Therefore, the heat generation rate inside the nanoparticle is

proportional to the nanoparticle volume, but, as $\sigma_{Abs}(\lambda)/V$ is maximum for small nanoparticle sizes, the photothermal effect can be maximized by increasing the number of nanoparticles in the point of interest [70].

The surrounding environment is heated through phonon-phonon relaxation on a scale of picoseconds while the lattice cools off, which can be achieved by using continuous wave lasers. Moreover, the lattice can be heated until particle melting or ablation by using high intensity laser pulses (figure 1.8) to induce cell damage through hyperthermia, coagulation and evaporation [73,74].

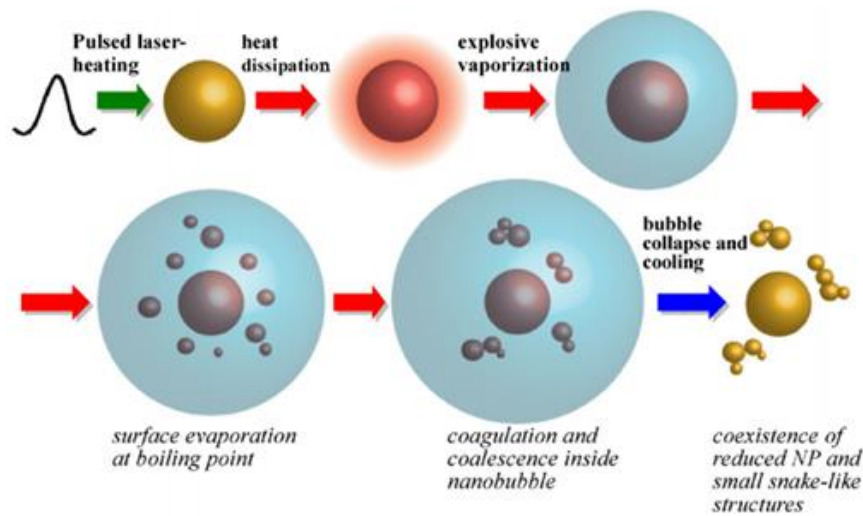


Figure 1.8. Schematic representation of nanoparticle heating processes induced by the incidence of nanosecond-pulsed-laser irradiation. Reprinted from [43].

The use of plasmonic nanoparticles allows photothermal therapy (PTT), which requires the use of gold nanoparticles that efficiently convert light into heat to induce localized temperature increase and, consequently, hyperthermia physiological responses that end into tumour cell death [75]. Moreover, it provides a means to photodynamic therapy that explores the generation of reactive-oxygen species (ROS) through excitation of a photosensitizer to induce cell death by apoptosis, thus depending on the availability of tissue oxygen [75].

1.3.4. Core/shell magnetic/plasmonic nanoparticles

Considering the bionanotechnological potential of gold nanoparticles, combination of optical and chemical properties of gold with magnetic nanoparticles affords a more versatile nanomaterial to tailor with the desirable properties owing to the ease of synthesis and ready functionalization *via* amine/thiol linkages of the gold shell, besides protecting the magnetic core from oxidation and corrosion [76,77]. Considering the diamagnetic contribution of the gold shell, the development of core/shell nanoparticles with relevant magnetic properties for biomedical applications requires a magnetic core with high magnetization, such as the transition metal ferrites (MFe_2O_4 , $M = Mn, Co, Ni$, etc.). The mild magnetic properties such as low coercivity and moderate magnetization, the good chemical stability and mechanical hardness, and the superparamagnetic behaviour contributes for the preferential use of these nanoparticles in drug delivery [78,79].

The unit cell of ferrite spinel structure is a cubic-closed-pack of oxygen ions formed by 32 O^{2-} anions with 8 tetrahedral positions and 16 octahedral positions occupied by cations (divalent or trivalent), where the small tetrahedral sites are occupied by the divalent M^{2+} ions and the large octahedral sites by the trivalent Fe^{3+} ions [62]. If the eight tetrahedral sites are occupied by M^{2+} ions, the ferrite is said to be normal spinel while the occupation of the octahedral sites is named inverse spinel. Generally, ferrites are ferrimagnetic materials, as the net magnetic moment results from the difference between the antiparallely oriented octahedral and tetrahedral moments [62]. Therefore, the adjustment of the M^{2+} ions chemical identity provides a means to modulate the magnetic properties.

Currently, there are several methods for the synthesis of transition metal ferrites, namely the co-precipitation of the M^{2+} and Fe^{3+} ions with a base in an aqueous solution or in a reverse micelle template, which is an inexpensive method, allowing large-scale production, but only with a moderate control in the properties and size distribution of the nanoparticles [80]. Another common method is the high-temperature organic phase decomposition of an iron precursor such as metal acetylacetonate.

Manganese ferrites stand out from the remaining ferrites owing to the higher magnetic susceptibility, besides being biocompatible and easily synthesized by co-precipitation without requiring calcination [62,81]. These advantages, coupled with the chemical and colloidal stability, make the manganese ferrite nanoparticles suitable for biomolecular magnetic labelling, drug delivery and contrast enhancement in magnetic resonance imaging [81].

The addition of a gold shell with different shapes and thickness will afford the magnetic properties of the manganese ferrites and gold's plasmonic properties in a single nanoparticle, further enhancing the therapeutic potential by combining different strategies such as photothermia, photodynamic therapy, magnetic hyperthermia, magnetic-guided delivery and magnetic resonance imaging contrast [77]. Considering the discussed effects of the shape on the plasmonic properties, in this work, two different geometries of shells will be investigated, the core/shell manganese ferrite/gold nanoparticles and the gold-decorated manganese ferrite nanoparticles (figure 1.9).



Figure 1.9. Schematic representation of the nanoparticles studied in this work, the manganese ferrite/gold core/shell nanoparticles and the gold-decorated manganese ferrite nanoparticles.

Moreover, the gold shell is expected to ensure diminished undesirable effects such as toxicity and tissue damage, and improve stability in physiological fluids and biocompatibility, though nanoparticles should have a size close to 50 nm, which allows them to cross fenestrations of the endothelial barrier of the vessels and to have a longer retention time in the interstices of the tumour cells [61].

1.4. Magnetogels

1.4.1. Concept and properties

Magnetogels ally the properties of both hydrogels and magnetic nanoparticles in a single system, introducing new properties that are unobtainable when the components are separated, besides the application of a magnetic field gradient allowing the control and targeting of the nanosystem to a specific location.

The hydrogels intertwined fibrillar structure encompasses microdomains of solvent pocket capable of retaining nanoparticles, which may interact with the fibers through non-covalent or covalent bonding, thus modifying network structure [29]. Such interactions provide control over the pristine materials properties, which can be translated into an enhancement of the adsorption or afford further mechanical, electrical, optical and thermal properties [29,82]. These properties ensure a means to tailor the nanomaterials

according to the desired application, i.e. can be adapted to mimic the structure of native tissues at the site of implantation and/or the efficient transport and bioaccumulation of an entity into a specific location, reducing side effects and enabling higher doses [27,83].

The nanoparticles might induce the formation of nucleation sites that work as cross-linkers and can be explored as a mean to regulate the porosity, swelling degree and gelation time [27]. Such nucleation sites are reported to enhance the swelling degree until a critical nanoparticle concentration, above which the nanoparticles impart less porosity to the network, that consequently leads to a lower swelling degree and affects positively or negatively the drug loading efficiency [84,85]. These mechanical and physicochemical changes introduced in the matrix can also be explored to stimulate cell adhesion, proliferation, differentiation and enabling visualization and *in vivo* follow-up through magnetic resonance imaging [27,86].

The mechanical and adsorption properties of magnetogels have also been explored on the development of materials for depollution due to the easiness of removal by a magnetic field, the large number of reusable cycles and the huge variety of removable contaminants [87-89]. Another industrial use has been the development of catalysts or biosensors using magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$) peroxidase-like activity [90-97].

From the biomedical perspective, magnetogels can be fabricated like the hydrogels into different types such as spheroid-like structures known as microgels, bead-based nanostructures, nanofibrous networks and mesh-like nanocomposites, which sometimes might require nanoparticle functionalization to grant stabilization, thus ensuring more control over the nanosystem, as the optimum structure can be adapted to the desired application (as discussed in section §1.2.2.) [90,98–104].

Moreover, the application of an alternating magnetic field provides control over the drug diffusion by the thermally induced network collapse and/or vibrational fiber disruption, while hydrogels rely only on the molecular diffusion and hydrogel degradation [105].

Overall, the magnetogels provide the possibility of combining a non-invasive detection of biomarkers at an early stage of a given disease with the choice, adoption and administration of medical personalized treatments, taking into account the genotype and the phenotypic characteristics of the patient by incorporating an immense variety of chemical and structural functionalities in the same architecture, i.e. creating theranostic agents [106,107].

Hereby, in this work, considering the gold shell added to the magnetic nanoparticles, two different hydrogelators were designed to avert the leaching out of the nanoparticles from the hydrogel matrix under an externally applied magnetic field owing to the S-Au and N-Au bond (figure 1.10).

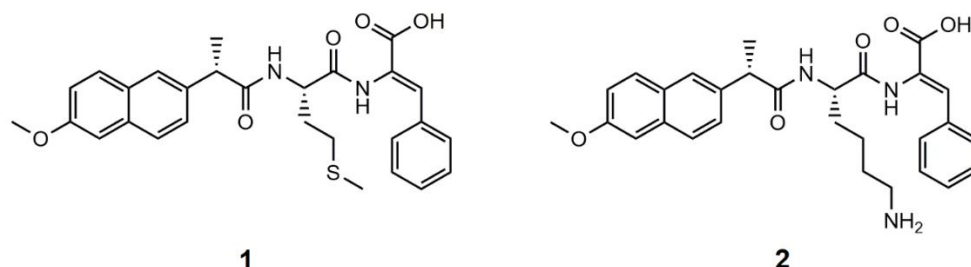


Figure 1.10. Schematic representation of the conceptualized and developed hydrogelators Npx-L-Met-ZΔPhe-OH (compound 1, MW = 506.62 g/mol) and Npx-L-Lys-ZΔPhe-OH (compound 2, MW = 503.60 g/mol).

The hydrogelators also contain the dehydroamino acid dehydrophenylalanine (ΔPhe) that endows the peptide hydrogelator with proteolytic stability and adds conformational restraints to the peptide backbone, favouring the self-assembly by enhancing the stacking of the aromatic moieties. On the other hand, the non-steroidal anti-inflammatory drug naproxen moiety not only affords aromatic groups for π-π stacking and other non-covalent forces required for hydrogelation, but also provides selectivity towards cyclooxygenase and ensures its inhibition [26,108,109]. Moreover, the presence of the naproxen moiety allows studying the gelation and other dynamic processes of hydrogels and magnetogels by exploring the intrinsic fluorescence of the naphthalene ring.

1.4.2. Magnetic hyperthermia

The alternating magnetic field (AMF) induced heating (magnetic hyperthermia) of a medium containing non-interacting magnetic nanoparticles, which magnetization varies linearly with the applied magnetic field, is associated with the delay of the magnetic nanoparticles' relaxation time compared to the oscillating AMF, where the dissipation power is expressed according to the equation [65,110]:

$$P = \mu_0 \pi \chi'' f H^2 \quad (1.6)$$

where H and f are the amplitude and frequency of the AC magnetic field, respectively, μ_0 is the vacuum magnetic permeability and χ'' is the AC magnetic susceptibility (imaginary part of the magnetic susceptibility), which the latter is expressed as [65]:

$$\chi'' = \frac{\omega \tau}{1 + (\omega \tau)^2} \chi_0 \quad (1.7)$$

where χ_0 is the actual susceptibility and τ is the total relaxation time, which is dependent on both the Néel and Brownian relaxation. The Néel relaxation is strongly size-dependent, occurring at sizes that only require a little bit of energy to induce rotation of the magnetic moment without changing the particle orientation, and has a characteristic relaxation time given by the equation [57]:

$$\tau_N = \tau_0 e^{\frac{KV}{k_B T}} \quad (1.8)$$

where τ_0 is a characteristic time of the material (10^{-9} – 10^{-12} s), K is the anisotropy constant, V the particle volume, k_B the Boltzmann constant and T the temperature.

The other relaxation mechanism is the Brownian relaxation, which pertains to the physical relaxation of the nanoparticles and is affected by medium viscosity. Here, the relaxation time is given by the following equation [57]:

$$\tau_B = \frac{3\eta V_H}{k_B T} \quad (1.9)$$

where η is the medium viscosity, V_H the particle hydrodynamic volume, k_B the Boltzmann constant and T the temperature.

As the Brownian relaxation is viscosity-dependent, the temperature associated medium viscosity decrease will enhance the Brownian relaxation rate, which is undesired so to avert free particle rotation due to viscosity fluctuation.

Magnetic hyperthermia has as main objective to expose the tissues to high temperatures (42–45 °C), either to kill tumor cells by apoptosis or to prompt higher susceptibility to radiation and antitumor drugs [57,111]. The thermal energy will promote denaturation of cytoplasmic, membrane and nuclear proteins required for DNA synthesis, thus a higher susceptibility of cancer cells to heat is induced by hindering the cellular division [57]. Nevertheless, a compromise around 42 °C is desired to avert affecting healthy cells. In fact, a reduced blood flux ($T > 42$ °C), combined with the depleted oxygen and nutrients levels, low pH, random vascularity and associated deficient heat dissipation, stimulates the sensitivity to hyperthermia, while a higher blood flux ($T < 42$ °C) increases oxygenation, drug accumulation, intracellular assimilation and DNA damaging, which affords a synergy with chemotherapy [57,62]. Therefore, the combined used of hyperthermia and chemotherapy will synergistically enhance the antineoplastic drugs cytotoxic effect and perfusion facilitation [111].

Magnetogels provide a highly advantageous platform for magnetic hyperthermia, as it reduces the requirement for repeated injections, owing to the long-term retention of the nanoparticles in the application site provided by the hydrogel matrix, and the prevention of local recurrence or metastasis through the combined controlled drug release of chemotherapeutic drugs with different cytotoxic pathways and toxicity profiles after tumor debulking surgery [105,112].

1.4.3. Magnetic resonance imaging

Magnetic resonance imaging (MRI) is an adequate imaging technique for diagnosis, owing to its high anatomical resolution with unlimited tissue depth and soft tissue contrast, thus overcoming the lack of sensitivity and unspecificity issues associated with the conventional contrast agents [113,114].

Besides magnetic hyperthermia, the magnetic nanoparticles can work as T2 contrast agents due to introduced local inhomogeneities, capable of passively accumulate in the tumour, due to the enhanced permeation and retention (EPR), and the physicochemical properties can be further modulated to actively direct to it, which is ideal to monitor diseases and detect injuries or deficiencies [113-116].

The magnetogels offer further improvements by exploring the proton spin dephasing (T2 relaxation) mechanisms: motional averaging regime (MAR), visit limited regime (VLR) or static dephasing regime (SDR) [116]. In the MAR regime, the distance between particles is smaller than the one travelled by the proton, which leads to the proton being subjected to different magnetic environments that might refocus it when an opposite polarity is found. Therefore, in this regime, full proton dephasing is not achieved, where the overall relaxation rate is retarded and proportional to particles size [116]. Above a critical nanoparticle size, the VLR regime occurs, which is characterized by the proton being fully dephased if it spends enough time in a full dephasing zone defined around the nanoparticle [116]. When the nanoparticle is large enough to the point that the proton is subjected to a constant magnetic environment, it is in the SDR regime, where full dephasing occurs in a single encounter [116].

The magnetogels fibres induce changes on the magnetic nanoparticles induced relaxation rates that might improve contrast if the nanoparticles are in the MAR regime, as the hydrogel network will slow down water self-diffusion. A longer proton retention time close to the particle surface will enhance MAR relaxation rate (higher contrast) by increasing the dephasing at each encounter, while the decrease in the exchange of protons between the inner and outer coating environment hinders the protons from reaching the full

dephasing zone, which will reduce relaxation rates (lower contrast) for the particles in the visit-limited regime [116].

1.5. Curcumin

In this work, the hydrogels and magnetogels potential as nanocarriers for controlled drug release was evaluated by encapsulating curcumin and studying the nanosystem interaction with small unilamellar vesicles (SUVs) used as biomembrane models.

Curcumin [1,7-bis(4-hydroxyl-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (figure 1.11) is a yellow-lipid soluble natural polyphenolic compound extracted from the roots of *Curcuma longa* [117,118].

Besides the use in food as a colorant and spice, it has been a molecule of intensive research owing to various beneficial biological and pharmacological activities, as it has been shown to regulate various pathways of the immune system, such as cell cycle checkpoints, apoptosis, antioxidant response, but also intracellular targets, including pathways and proteins that control tumour progression [118].

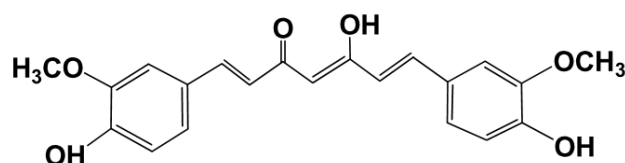


Figure 1.11. Molecular structure of curcumin (MW = 368.37 g/mol) [119].

However, curcumin exhibits low solubility in water at acidic and neutral pH, low stability at basic pH, and requires organic solvents such as acetone, hexane, methanol and ethanol to solubilise it [119]. Such physicochemical aspects highly affect its absorption and stability in the gastrointestinal tract. Therefore, the low absorption, rapid metabolism and systemic elimination, which together with low solubility and degradation in water, the need to use cosolvents and uncontrolled precipitation restricts the use of curcumin in the treatment of cancer and other applications [118].

Hereby, the encapsulation of curcumin in hydrogels and magnetogels is expected to improve the water solubility, stability and bioavailability of this compound, which has advantageous properties for antioxidant, antitumour and anti-inflammatory applications. From the photophysical point of view, curcumin is a fluorescent compound highly sensible to polarity, which is very weak in aqueous medium. Moreover, the low energy π - π^* excitation is allowed due to the intramolecular exciton coupling between the electric dipole transition moments of the two conjugated feruloyl chromophores [117], which can be

explored to follow its location in the hydrogel/magnetogel, changes introduced by the nanoparticles and the interaction with biomembrane models.

1.6. References

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Chapter 2 – Analysis and characterization techniques

In this chapter, the techniques required for the analysis and characterization of the developed nanosystems will be initially described, for a better understanding of their principles and comprehension of upcoming results in chapter 4.

2.1. Electromagnetic radiation

The electromagnetic radiation is described as a form of energy propagation in space, which contains wave and particle characteristics. From the wave point of view, the electromagnetic radiation behaves as a wave comprising an electric and magnetic fields, which vectors oscillate perpendicularly and sinusoidally along the wave propagation direction [1].

The electromagnetic waves are described by a wavelength (λ), period (T), frequency (f), amplitude and velocity (ν), where the velocity attains its maximum value of 2.9971×10^8 m/s (light speed, c) in vacuum and relates with frequency and period through the following equations [1]:

$$c = \lambda f \quad (2.1)$$

$$f = \frac{1}{T} \quad (2.2)$$

Although the wave description of electromagnetic radiation is useful for the understanding of reflection, refraction, interference, diffraction and polarization phenomena, such is not adequate for the description of light absorption and emission. Hereby, a corpuscular description was required, where the radiation is constituted by light particles, the photons, whose energy relates to the wave frequency and wavelength by the equation:

$$E = hf = \frac{hc}{\lambda} \quad (2.3)$$

where h represents the Plank's constant (6.6256×10^{-34} J.s) [1]. Therefore, the wave-particle duality appears, where the electromagnetic radiation behaves as a wave when it propagates in space, but as a particle when it interacts with matter.

2.1.1. UV-visible light absorption

The UV-visible electromagnetic waves include the wavelength range from 190 nm to 750 nm. Upon its incidence on a medium (solid, liquid or gaseous), the photons with the energy (i.e. frequency) equal to the energy difference between the ground and excited states of the molecular species are absorbed, apart from occurring a reduction on intensity [2]. Moreover, the total energy of molecules is described by the electronic, vibrational and rotational energy contributions. Hereby, the electronic transition, at room temperature and with no perturbations, consists on the promotion of an electron from the lowest vibrational level of a molecular orbital ground state to the various rotovibrational (vibrational and rotational) levels of the unoccupied molecular orbital. Such effect is translated in the appearance of various peaks in the absorption spectrum where the different wavelengths correspond to transitions requiring different energy. Furthermore, the peaks show a broadening as a result of the transition to the various rotovibrational levels (homogeneous broadening), which can also be affected by conditions such as the fluctuation of the structure of solvent shell (inhomogeneous broadening) [2].

The electronic transition can be classified according to the involved molecular orbitals, which can be bonding (σ or π), antibonding (σ^* or π^*) or non-bonding (n). The σ orbitals can be due to the overlap of two s atomic orbitals, one s and one p atomic orbitals, or two colinear p atomic orbitals, while a π orbital results from the lateral overlapping of two p atomic orbitals. The n orbitals are associated to the non-bonding electrons of heteroatoms such as oxygen or nitrogen. Figure 1 shows the possible molecular orbital transitions, where the energy increases from the $n \rightarrow \pi^*$ to $\sigma \rightarrow \sigma^*$. The $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ are the transitions commonly detected by the spectrophotometer, as their energies occur in the UV-visible region, while the $\sigma \rightarrow \sigma^*$ and $\pi \rightarrow \sigma^*$ require vacuum-UV radiation.

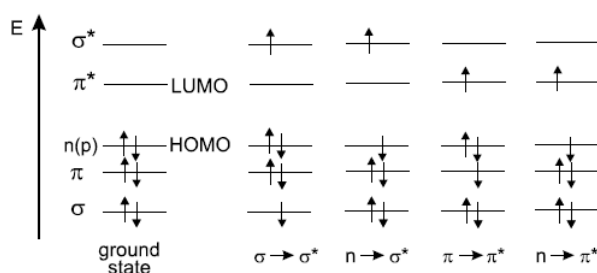


Figure 2.1. Scheme of the molecular orbital energy levels (HOMO: Highest Occupied Molecular Orbital; LUMO: Lowest Unoccupied Molecular Orbital) and possible electronic transitions. The molecular orbitals can be bonding (σ , π), antibonding (σ^* e π^*) or non-bonding (n) [2].

Apart from the electronic transition to a molecular orbital of higher energy, the electrons might attain a singlet or triplet excited state. If an electron belonging to a filled molecular orbital in the ground state is promoted and the spin remains unchanged, the total spin quantum number ($S = \sum s_i$, with $s_i = -\frac{1}{2}$ or $+\frac{1}{2}$) and the multiplicity ($M = 2S + 1$) will remain equal to zero and one, respectively [2]. Thus, the ground and excited states are said to be in a singlet state (S_0) or singlet excited state (S_n). However, if the spin of the promoted electron changes, the total spin quantum number becomes 1, as there are two parallel electron spins, and the multiplicity changes to three, thus transitioning to a triplet excited state (figure 2.2), which according to Hund's Rule has lower energy than the singlet state of the same configuration [2].

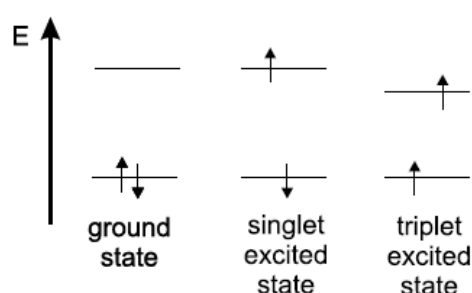


Figure 2.2. Scheme representing the difference between singlet and triplet states. If the excited molecule shows a total spin quantum number equal to zero, the multiplicity is 1, thus it is in a singlet excited state, if the multiplicity is 3, it is said to be in a triplet excited state [2].

Besides the available electronic states, the transitions are subjected to selection rules, mainly by the spin- and symmetry-forbidden transitions. According to the first rule, the transition between different multiplicity states is forbidden (low probability), but there is always a weak interaction between wavefunctions of different multiplicity via spin-orbit coupling that varies with the fourth power of the atomic number [2]. The second rule implies that transitions between molecular orbitals with different symmetry are forbidden due to the poor spatial overlap of the molecular orbitals, i.e. the $n \rightarrow \pi^*$ transitions are forbidden. However, due to the molecular vibrations causing some departure from the perfect symmetry (vibronic coupling), the symmetry-forbidden transitions can also be observed [2]. The probability relates with the intensity, where the most allowed transitions have higher probability, thus originating more intense bands while the forbidden transitions will have lower intensity due to lower probability.

Moreover, the environment conditions, such as pH and solvent polarity, can affect the electronic transitions. The solvent polarity favours the stabilization of the n molecular orbitals, through interaction with the non-bonding electrons of the heteroatoms, over the antibonding orbitals π^* , which are more

stabilized than the π orbitals. Hereby, the increase in solvent polarity will produce a blue shift of the $n \rightarrow \pi^*$ transitions, while a red shift will be observed for the $\pi \rightarrow \pi^*$ transitions.

2.1.1.1. Lambert-Beer's law

Experimentally, the absorbed radiation intensity by an absorbing medium is expressed as the measure of absorbance, A_λ , or transmittance, T_λ , at a wavelength λ [2]:

$$T_\lambda = \frac{I_\lambda}{I_\lambda^0} \quad (2.4)$$

$$A_\lambda = -\log(T_\lambda) \quad (2.5)$$

where I_λ^0 is the incident light intensity and I_λ is the transmitted light intensity by the sample. The incident light decays exponentially when traversing an absorbing medium, according to the equation [2]:

$$I_\lambda = I_\lambda^0 e^{-\varepsilon_\lambda bc} \quad (2.6)$$

From the previous expressions, it is possible to deduce the Lambert-Beer's law [2]:

$$A_\lambda = \varepsilon_\lambda bc \quad (2.7)$$

which relates the absorbance by a homogeneous sample at a given wavelength and solvent with its concentration, c , the distance of the absorbing sample travelled by light, b , and the molar absorption coefficient, ε_λ , that expresses the efficiency (probability of occurring the electronic transition) of light absorption. However, the Lambert-Beer's law is only valid when using a monochromatic radiation and measuring a diluted solution containing a single absorbing species (thus, a single molar absorption coefficient), as for concentrated solutions, the linearity between absorbance and concentration might be lost [2,3]. If more than one absorbing species is present, the absorbance will be the sum of all the absorbances by each species.

As solvents might absorb in the desirable region, such is an important parameter to consider before setting the experiment, as it must be transparent in the wavelength region where the absorbance by entity of interest will be measured. Hereby, it is important to remove the absorption of the medium by using the intensity of the monochromatic radiation transmitted from the medium without the entity of interest, instead of I_λ^0 [2]:

$$(A - A_S)_\lambda = \log \left(\frac{I_\lambda^0}{I_\lambda} \right) - \log \left(\frac{I_\lambda^0}{I_\lambda^S} \right) = \log \left(\frac{I_\lambda^S}{I_\lambda} \right) \quad (2.8)$$

where I_λ^S is the radiation intensity transmitted by the medium without the identity of interest (blank).

2.1.1.2. Spectrophotometer

The electronic absorption spectrum is measured with a spectrophotometer, which can be either single or double beam. In a single beam spectrophotometer, the absorbance is measured by comparing the relative light intensities before and after measuring the sample, thus requiring an initial measurement of the solvent absorption before measuring the sample. The double beam spectrophotometer is similar to the single beam, but has the advantage of comparing the light intensity of two lights paths, one with the sample and the other containing only the medium, which allows accounting for any solvent absorbance variation due to temperature fluctuation during the measurements.

The spectrophotometer is essentially constituted by a beam source, a monochromator, beam-splitter, the sample holder, rotating disc, detector (photomultiplier) and the computer, which allows the processing and acquisition of information further translated into a graphical representation of the absorbance variation along the selected wavelengths [3].

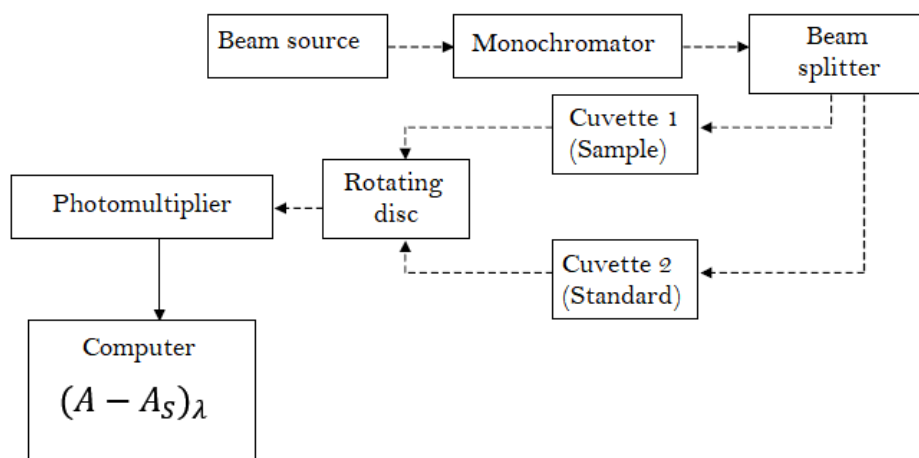


Figure 2.3. Schematic representation of the spectrophotometer. Adapted from [3].

2.1.2. Luminescence

Luminescence is described as the emission of photons in the ultraviolet, visible or infrared region by species previously electronically excited, which can be organic, inorganic or organometallic [2]. The excitation is consequence of the interaction between the electromagnetic radiation and matter, which further leads to the classification of luminescence according to the type of molecular excitation. Fluorescence and phosphorescence are particular cases of luminescence, where the excitation results from the absorption of photons by a species and, subsequently, its excitation to an electronic excited state, which de-excitation proceeds through the emission of photons, i.e. photoluminescence [2].

The signal (absorption, emission or scattering) from the interaction between radiation and matter is then translated into a spectrum as a function of an electromagnetic wave characteristic, such as frequency, energy, wavelength and wavenumber.

2.1.2.1. Intramolecular relaxation mechanisms

Once the molecule has been excited to an excited state, the associated instability to the state leads the molecule to relax to the ground state, which can happen through different photophysical and photochemical pathways (figure 2.4).

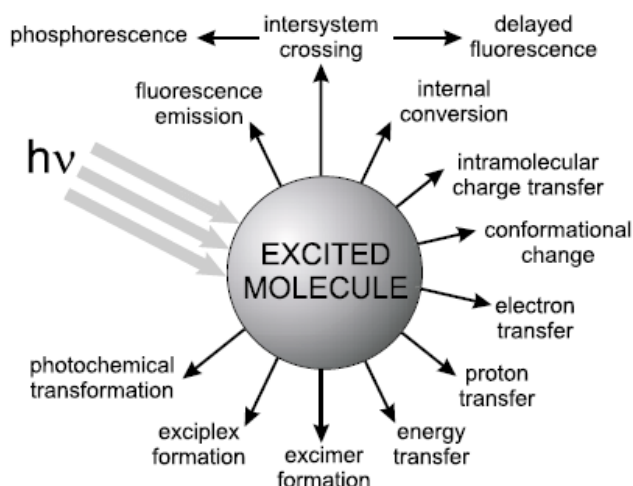


Figure 2.4. Schematic representation of the possible de-excitation pathways of an excited molecule [2].

The photophysical pathways can be classified into radiative, when the relaxation occurs through emission of photons, or non-radiative if occurs through collision with solvent molecules. Fluorescence is a radiative photophysical process (emission of photons), which generally occurs through the transition from the

lowest vibrational level of the first singlet excited state (S_1) to the different vibrational levels of the ground state (S_0). The photon emission is as fast as the absorption. However, the molecule keeps in the excited state for a certain period before de-excitation (10^{-9} - 10^{-12} s) through photon emission or before other excitation processes [2]. Nevertheless, the fluorescence will occur only if it is the process that grants the lowest excited state lifetime.

The non-radiative relaxation processes include the internal conversion, vibrational relaxation and intersystem crossing. The internal conversion consists in the non-radiative transition between two electronic states of the same multiplicity, which can be followed by vibrational relaxation to the lowest vibrational level of the next electronic state. The vibrational relaxation can also occur when the molecule is excited to a higher vibrational level than the lowest vibrational level of the excited electronic state in a scale time of 10^{-13} - 10^{-11} s [2]. The existence of isoenergetic vibrational levels belonging to electronic states of different multiplicity allows the occurrence of the intersystem crossing phenomenon between a singlet and triplet excited state (e.g. between the S_1 and T_1 excited states) which can compete with fluorescence, as it has a time scale of 10^{-7} s to 10^{-9} s [2]. Moreover, the intersystem crossing is favoured in the presence of heavy metals due to the spin-orbit coupling. The relaxation from the lowest vibrational level of the first triplet excited state to the ground state can occur through the emission of photons, the process being called phosphorescence. However, as the transition between states of different multiplicity is forbidden (low probability), at room temperature, the non-radiative phenomena predominate, while at low temperatures and/or in a rigid system, it is possible to enhance the relaxation through phosphorescence due to the reduction of molecular collisions [2]. The above-mentioned relaxation processes are represented in the Perrin-Jablonski diagram in figure 2.5.

The non-radiative processes also include the delayed fluorescence activated by temperature and by triplet-triplet annihilation, apart from the intersystem conversion from T_1 to S_0 . When the energy difference between the S_1 and T_1 electronic states is small or when the T_1 lifetime is long, the emission of delayed fluorescence thermally activated can occur, where the emission shows the same spectral distribution at the "normal" fluorescence, but with efficiency proportional to temperature and a larger decaying time constant due to the molecule remaining in the triplet state before relaxing through S_1 [2]. On the other hand, the delayed fluorescence by triplet-triplet annihilation occurs in concentrated solutions, due to the possibility of collision between molecules in T_1 state provide the required energy to send the molecule back to the S_1 state, thus occurring the emission of fluorescence with a decaying time constant equal to half the triplet state lifetime of a diluted solution [2].

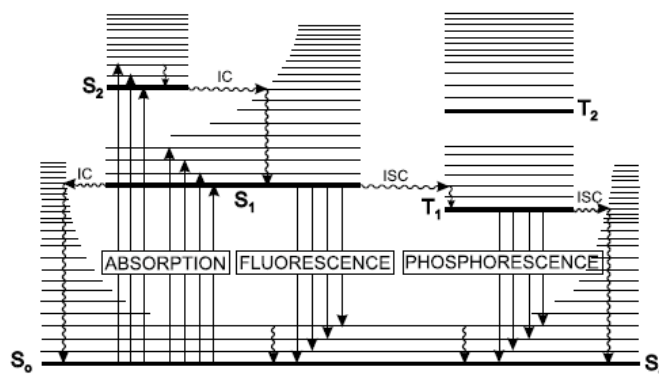


Figura 2.5. Representation of the Perrin–Jablonski diagram that summarizes the common photophysical processes after absorption: fluorescence, phosphorescence, internal conversion (IC), intersystem conversion (ISC) and vibrational relaxation [2].

Due to the low internal conversion efficiency, fluorescence tends to occur naturally from the lowest vibrational level of the first excited state to the various vibrational levels of the ground state. Therefore, in a fluorescence spectrum the peaks corresponding to the electronic transitions appear at higher wavelengths than absorption peaks owing to the loss of vibrational energy. Moreover, the shift associated with the 0-0 transitions of absorption and fluorescence spectra are caused by the solvent effect, where the de-excitation happens from a lower energetic S_1 state that is in equilibrium with the solvent (after its reorientation) to a S_0 state not in equilibrium [2]. Hereby, the first fluorescence peak does not coincide with the 0-0 transition of absorption spectrum as the energy gap of the former is smaller.

By exploring all the above processes described, the high sensitivity, specificity and the possibility of providing spatial and temporal information has made fluorescence a fundamental tool to study the structure and dynamics of matter [1].

2.1.2.2. Fluorescence quantum yield

The fluorescence quantum yield (Φ_F) is the fraction of excited molecules that relax to the ground state (S_0) through photon emission, i.e. is the ratio of photons emitted per photons absorbed by a sample. The value can be obtained by comparing with a reference compound and is defined by the equation [2]:

$$\Phi_F = \frac{k_r^S}{k_r^S + k_{nr}^S} = k_r^S \tau_S \quad (2.9)$$

which relates the fluorescence quantum yield with the radiative (k_r^S) and non-radiative (k_{nr}^S) rate constants and the excited state lifetime (τ_S).

The quantum yield is an important fluorescence parameter, as the higher its value the easier is the detection of the fluorescent compound. As the excited molecules remain in the excited state for a reasonable time, interaction with other molecules might happen that compete with the relaxation. These processes can result in fluorescent species which emission overlaps the initially excited molecule [2]. Besides that, its value can be affected by different factors, such as pH, polarity, temperature, hydrogen bonding and quenchers (figure 2.6) [2].

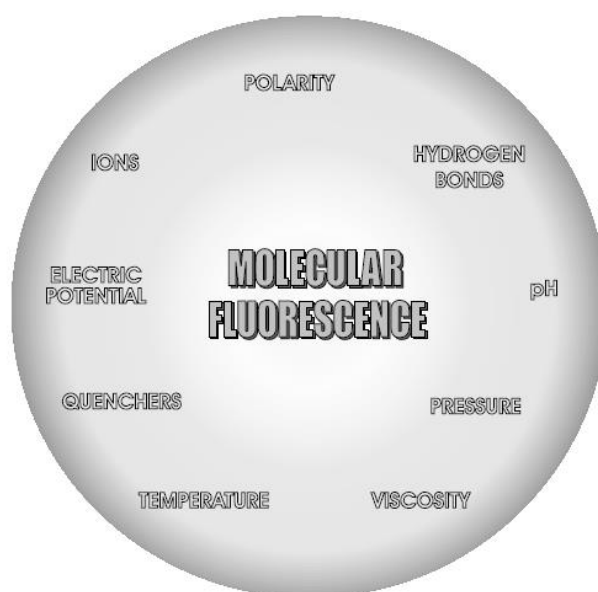


Figure 2.6. Representation of the parameters that might influence the fluorescence emission [2].

From the equation 2.9, it is also observed that the fluorescence quantum yield is proportional to the excited state lifetime. However, such proportionality is only valid if the de-excitation pathways do not include the interaction with other molecules. Some examples where the fluorescence quantum yield is affected without changing the excited state lifetime is the static fluorescence inhibition by non-fluorescent complexes formation or through collision with oxygen, which will affect compounds with long lifetimes as the inhibition is controlled by diffusion. The temperature can also lead to a fluorescence decrease of quantum yield due to non-radiative processes. On the other hand, the reduction of temperature can reduce the de-excitation through collision and increase phosphorescence, as it has a long lifetime. Hereby, the fluorescent characteristics (spectrum, quantum yield and lifetime) are affected by any process that includes the interaction of the excited molecule with the vicinity [2].

2.1.2.3. Fluorescence anisotropy

In natural light, the electric and magnetic fields oscillate perpendicularly to the direction of propagation, without a preferential orientation. However, the use of a polarizer allows attaining polarized light, where the electric field oscillates in a defined orientation. The incidence of polarized light will preferentially excite the molecules with transition moments orientation close to the light polarization plane, which is called the photoselection effect [2].

However, if fluorophores diffuse freely, are bonded to small molecules or have a fast rotation movement, the initial orientation will be lost before emission and fluorescence polarization will be small. Thus, the fluorescence depolarization is a consequence of the fluorophore rotational movement, which is itself affected by the medium viscosity (η), the systems dimensions and excited state lifetime (τ_s) according to the equations:

$$\frac{1}{r} = \frac{1}{r_0} \left(1 + \frac{\tau_s}{\tau_c}\right) \quad (2.10)$$

$$\tau_c = \frac{V_H \eta}{k_B T} \quad (2.11)$$

where r_0 and r are the intrinsic and measured fluorescence anisotropy, respectively, τ_c is the rotational correlation time and V_H is the molecule hydrodynamic volume.

Considering the above mentioned characteristics, fluorescence anisotropy can provide information on the molecular mobility, size, shape, flexibility and medium fluidity. Theoretically, the anisotropy of a system where no rotational movement occurs (fundamental or intrinsic anisotropy) can have values of 0.4 or -0.2 for transition moments parallel and perpendicular to the light polarization plane, respectively [2].

Experimentally, the anisotropy value, r , can be calculated from the equation [2,4]:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (2.12)$$

where I is the fluorescence emission intensity, the subscript V corresponds to the polarizer vertically oriented and H to the polarizer horizontally oriented. The subscripts VV and HH stand for excitation and emission parallelly polarized, the subscripts VH and HV correspond to excitation and emission perpendicularly polarized and $G = \frac{I_{HV}}{I_{HH}}$ is the instrumental correction factor.

2.1.2.4. Förster Resonance Energy Transfer (FRET)

Förster Resonance Energy Transfer (FRET) technique has been an important tool in the observation of isolated events such as the proteins activity, interactions between cells or molecules [5]. FRET is a non-radiative energy transfer through long range dipole-dipole interactions between a donor and an acceptor molecule (both in singlet state), where the acceptor emits fluorescence without being directly excited, i.e. only the donor is excited, and the acceptor attains the excited state while the donor relaxes back to the ground state. Such process can only occur if the donor emission spectrum overlaps the donor absorption spectrum, such that occur some donor vibronic transitions with the same energy of the acceptor (figure 2.7.) [2,4,5]. Moreover, the process requires a proper orientation of the fluorophores dipole moments and a distance between 10 and 100 Å [2].

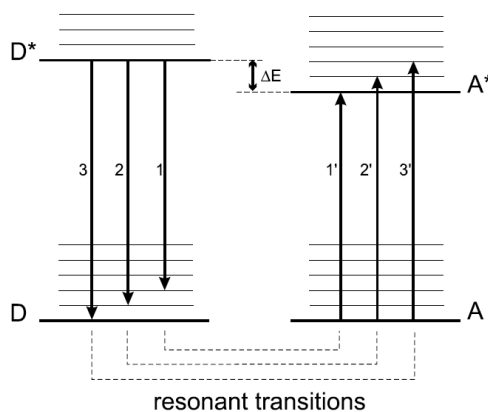


Figure 2.7. Schematic representation of the donor and acceptor molecules coupled transitions [2].

The FRET rate constant is defined by [2,4,5]:

$$k_{FRET} = k_D \left[\frac{R_0}{R_{DA}} \right]^6 \quad (2.13)$$

where k_D is the donor emission rate constant without the presence of an acceptor, R_{DA} is the distance between the donor and acceptor, R_0 is the critical distance, i.e. the distance at which both phenomena (donor fluorescence emission and FRET) are equally probable. The transfer efficiency can be defined by [2,4,5]:

$$\Phi_{FRET} = \frac{k_{FRET}}{k_D + k_{FRET}} \quad (2.14)$$

which can also be expressed as [2,4,5]:

$$\Phi_{FRET} = 1 - \frac{I_{DA}}{I_D} \quad (2.15)$$

where I_{DA} and I_D are the donor emission intensities in the presence and absence of an acceptor, respectively [1]. The above-mentioned equations for FRET efficiency and the energy transfer rate constant can be combined to obtain a relation between the donor and acceptor distance, R_{DA} , and FRET efficiency [2,4,5]:

$$\Phi_{FRET} = \frac{1}{1 + (\frac{R_{DA}}{R_0})^6} \quad (2.16)$$

The distance R_0 , at which the FRET efficiency is 50% [1], can be determined by the equations [2,4,5]:

$$R_0 = 0.2108[\kappa^2 \phi_D n^{-4} J(\lambda)]^{1/6} \quad (2.17)$$

$$J(\lambda) = \int_0^\infty I_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda \quad (2.18)$$

where κ^2 is an orientational factor ($=2/3$), ϕ_D is the donor fluorescence quantum yield without acceptor, n is the medium refractive index, $I_D(\lambda)$ is the normalized donor fluorescence spectrum so that $\int_0^\infty I_D(\lambda) d\lambda = 1$ and $\varepsilon_A(\lambda)$ is the acceptor molar absorption coefficient.

2.1.2.5. Spectrofluorometer

Experimentally, the spectrofluorometer is the equipment used to measure the fluorescence intensity (I_F) in the steady-state according to the equation [2]:

$$I_F = I_0(1 - 10^{-\varepsilon_{exc}bc})\phi_F F_{\lambda_{em}} d_{\lambda_{em}} \quad (2.19)$$

where I_0 is the incident radiation intensity, $(1 - 10^{-\varepsilon_{exc}bc})$ the absorbed radiation fraction, ϕ_F the fluorescence quantum yield, $F_{\lambda_{em}}$ the emitted light fraction with wavelength λ_{em} , and $d_{\lambda_{em}}$ is an instrumental factor that represents the light fraction with wavelength $\lambda = \lambda_{em}$ detected.

This equipment is generally constituted by a xenon arc lamp that continually emits from 250 nm to the infrared region. The light then passes through a monochromator, where the excitation wavelength is selected. The fluorescence emitted by the sample is measured at right angles to the excitation light to reduce the interference of the transmitted light from the sample. After going through a second monochromator, the light is detected at a photomultiplier and the signal is processed in the computer.

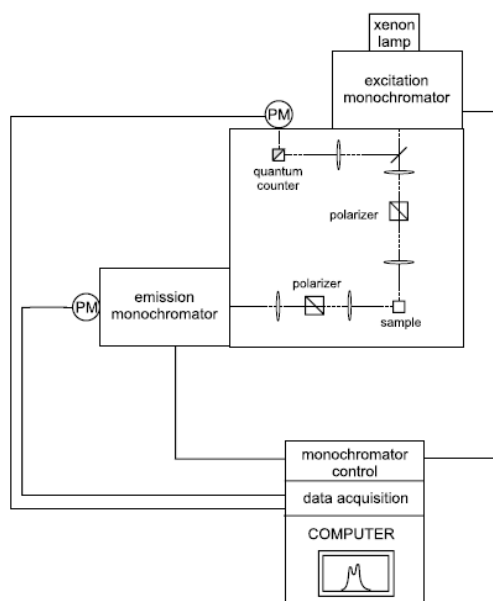


Figure 2.8. Schematic representation of a conventional spectrofluorometer [2].

2.1.3. FTIR technique

Upon the absorption of infrared radiation, the molecule is excited to higher vibrational energy states where the interaction of radiation with matter leads to changes in molecular dipoles [6-8], i.e. the molecule is only promoted to the excited state if its dipole moment (μ) changes during the vibration. Thus, the presence of symmetry elements might lead to vibration modes that are forbidden or degenerate [7]. Hereby, in general a molecule with N atoms has $3N$ degrees of freedom, where three consist on the translational motion and other three correspond to the rotation motion about the cartesian axis. Therefore, a nonlinear molecule has $3N-6$ degrees of freedom, while a linear one has $3N-5$ possible vibrational modes.

Although each vibrational mode of the atoms can be approximately described by a harmonic displacement from their equilibrium position (figure 2.9) [7,8], in practice the potential energy variation of the atoms displacement is not parabolic, thus being best described by an anharmonic potential function (Morse-type), which can firstly approximate the vibrational energy states V_{iv} according to the equation [7]:

$$V_{iv} = h\nu_i \left(v_i + \frac{1}{2} \right) + h\nu_i x_i \left(v_i + \frac{1}{2} \right) \quad (2.20)$$

where h is the Planck's constant, ν_i is the modes fundamental frequency, v_i is the i -th mode vibration quantum number, and x_i is a dimensionless anharmonicity constant that according to the mode varies

between -0.001 and -0.02 [7]. Nevertheless, the fundamental transition ($\Delta v_i=1$) can be approximated by the simple harmonic motion that corresponds to the first term of the equation 2.20, thus only requiring the anharmonicity term for higher quantum levels, such as overtones ($\Delta v_i>1$) [9]. From the Hooke's law, the fundamental frequency is related to the mass of bonded atoms and strength of the covalent bond according to the equation [9]:

$$\nu = \frac{1}{2\pi c} \sqrt{\frac{k}{\mu}} \quad (2.21)$$

where k is a force constant, c the speed of light, and μ the reduced mass.

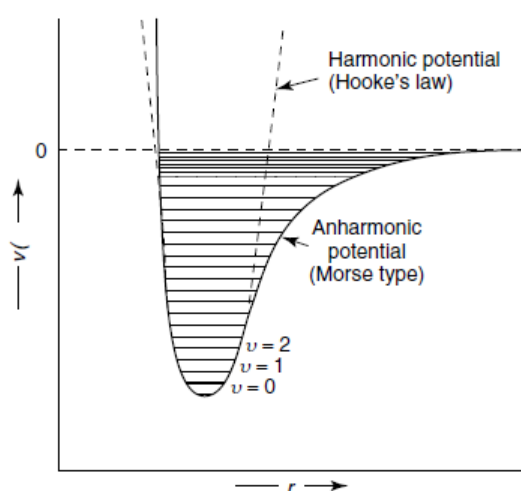


Figure 2.9. Graphical representation of the potential energy variation of a diatomic molecule as a function of the atoms distance [7].

Vibration modes (figure 2.10) can involve changes in bond length (stretching) and angle (bending), while being in-phase (symmetrical), out-of-phase (asymmetrical), in-plane or out-of-plane. Moreover, the intensity of infrared bands is governed by the square of the dipole moment change along the vibration, which leads to the very polar bonds (C-O, C=O and C-F) being more intense than less polar bonds (C-C, C-S, S-S) [7].

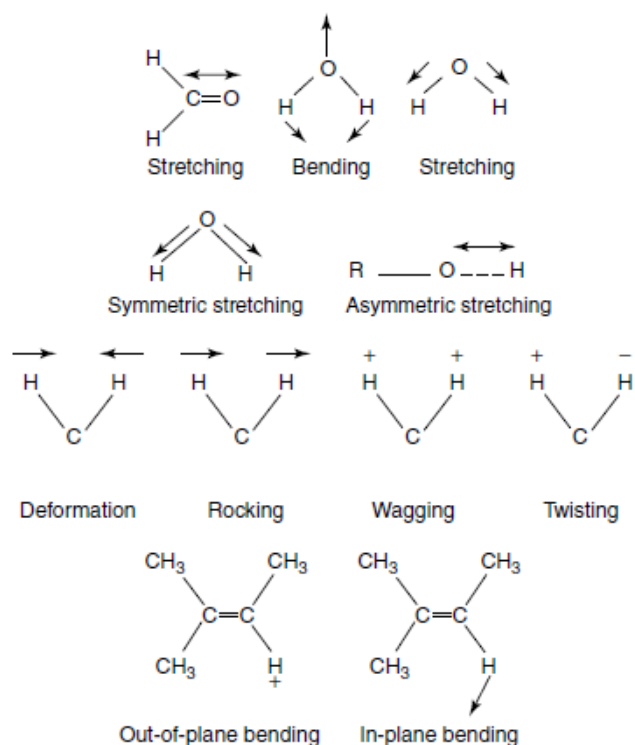


Figure 2.10. Schematic representation of the possible vibration modes [8].

Hereby, since the vibrational frequency depends on the strength and polarity of the bonds, such implies that every intra- or intermolecular effect, such as the atoms mass, the bond type, withdrawing and donor effects, coupling with other vibrations, bond angles and conformation, hydrogen bonding, electric fields and conformational freedom will affect the vibrational frequency [6,10].

2.1.3.1. FTIR-ATR spectrometer

Currently, the infrared spectrometers make use of a Michelson interferometer (figure 2.11), where light from an infrared source is collimated and directed to a beam splitter that reflects half the incident light to a fixed mirror and the other half to a movable mirror, from which only half of the light reflected back to the beam splitter by the mirrors passes to reach the detector. Upon beam recombination, constructive or destructive interference occurs, thus generating a signal that varies with as a cosine function of the optical path difference. The signal is then processed in the computer by performing the Fourier transform, which generates a spectrum $S(\lambda)$.

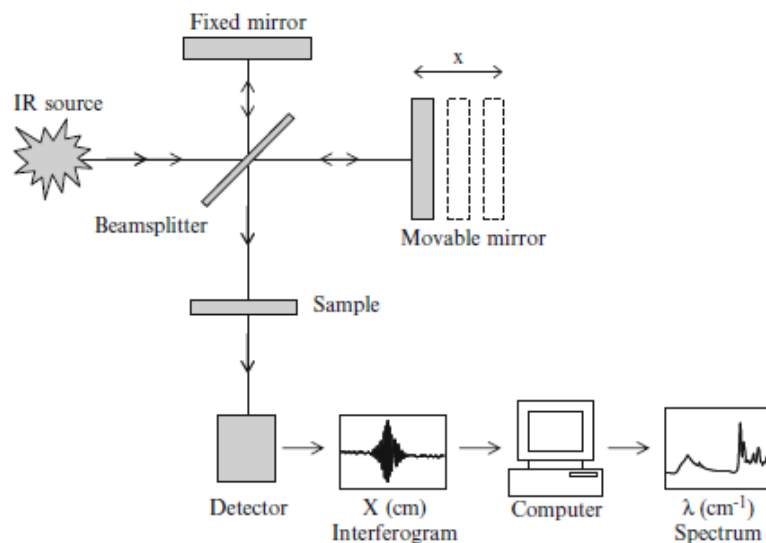


Figure 2.11. Schematic representation of the Fourier transform infrared spectrometer [11].

The spectrum can be obtained by transmission, where the infrared light passes through a cuvette containing the sample or alternatively by the attenuated total reflectance technique. In the latter, the sample is placed on a crystal (e.g. zinc selenide, thallium iodide/thallium bromide and germanium) with an index of refraction higher than the sample, generally larger than 2 [12]. The incidence of infrared radiation is done at a critical angle:

$$\theta_c = \sin^{-1} \frac{n_2}{n_1} \quad (2.22)$$

where n_1 and n_2 are the refraction index of the crystal and sample, respectively. Such incidence geometry allows the total and repetitive reflection of infrared radiation at the interface crystal/sample, thus creating an evanescent wave along the interface that is attenuated due to the absorbance by sample (figure 2.12).

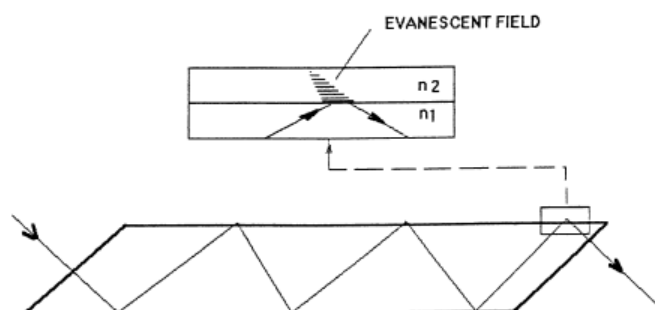


Figure 2.12. Schematic representation of the multiple infrared radiation reflections undergoing inside the crystal [12].

The penetration depth of the sample by the evanescent wave is defined as the depth at which the evanescent wave is attenuated to 36.8% of its total intensity and is given by the equation [12]:

$$d_p = \frac{1}{2\pi\bar{\nu}n_2\sqrt{(\sin^2 \theta - n_{sc}^2)}} \quad (2.23)$$

where $\bar{\nu}$ is the wavenumber, n_2 is the crystal refractive index, θ is the angle of incidence, n_{sc} is the ratio $\frac{n_2}{n_1}$. From the equation above, it is observed that d_p varies inversely with the wavenumber, which results in more intense signals at low wavenumbers [12]. Moreover, considering that the absorption of radiation is governed by the real and imaginary complex refractive index, opposed to the transmission mode that is dominated by the imaginary component, the ATR technique is controlled by both optical constants [7].

2.1.4. Nuclear magnetic resonance (NMR) spectroscopy

Under the application of an intense magnetic field, the atomic nuclei develop energy states where the energy difference falls in the radio-frequency region, 10 MHz – 1 GHz. The charged spinning nucleus develops a magnetic field characterized by a magnetic moment that relates to the spin angular momentum vector according to the equation [13]:

$$\mu = \gamma I \quad (2.24)$$

where γ is the gyromagnetic ratio, which is positive for magnetic moments parallel to the spin angular momentum (I) and negative for the opposite. The application of a strong magnetic field removes the energy degeneracy of the nucleus $2I+1$ possible orientation, where the energy relates with the magnetic field (B), magnetic quantum number (m) and Planck's constant (radians), according to the equation [13]:

$$E = m\hbar\gamma B \quad (2.25)$$

Hereby, a 1/2 nucleus spin only has two energy states, which energy gap can be expressed in frequency, according to the equation [13]:

$$f = \frac{\gamma B}{2\pi} \quad (2.26)$$

From the equation 2.26 it is possible to observe that a positive gyromagnetic ratio (^1H and ^{13}C) will result in the spin up orientation having the lower energy and vice-versa. From a classical perspective, the applied field is proportional to the precession (Larmor) frequency that resonates with the spinning nucleus. Another consequence of the application of a magnetic field is the occurrence of a Boltzmann distribution of the magnetic moment population, according to the equation [13]:

$$\frac{N_{upper}}{N_{lower}} = e^{-\frac{h\nu B}{2\pi kT}} \quad (2.27)$$

According to equation 2.27, for a positive gyromagnetic ratio, there is a small excess of magnetic moments aligned with the applied magnetic field and, thus, the net magnetization occurs along the z+ direction [13]. The application of a radio-frequency equal to the spins Larmor frequency will result in the resonance between the rotating magnetic field and the precessing spins, which will feel a steady magnetic field and the magnetization will precess around its direction [13]. Hereby, if a pulse is applied the magnetization will precess into the xy plane at the Larmor frequency and a signal will be induced in a coil that can be amplified and processed.

2.1.4.1. Nuclear environment effect

The subjection to a magnetic field of the nuclei spins that have different chemical environments will result in nuclei with different Larmor frequencies even if they are the same element [13,14]. Hereby two effects arise, the chemical shift and the spin-spin coupling which are dependent and independent on the magnetic field strength, respectively. The former is associated with the magnetic field sensed by the nucleus (B) being smaller than the applied magnetic field (B_0) owing to an opposing magnetic field (B') created by the surrounding electrons, which can be related to a shielding constant (α) according to the following equation [13]:

$$B = B_0 - B' = B_0(1 - \alpha) \quad (2.28)$$

Consequently, the nucleus within the atom resonates at a lower frequency than a bare nucleus, where resonance can be attained either by varying the frequency with a constant magnetic field, or by screening the magnetic field while holding the frequency constant [13]. However, the measure of the absolute chemical shift is inappropriate and difficult to determine, what led to the expression of the chemical shift (δ) in terms of the difference between the frequency of interest (ν_s) and a reference nucleus (ν_{ref}) as expressed in the following equation [13]:

$$\delta = \frac{(\nu_s - \nu_{ref})}{\nu_{ref}} \quad (2.29)$$

where the chemical shift is quoted in parts per million and is independent of experimental conditions. On the other hand, the interaction between not equivalent magnetic moments of neighbouring nuclei will lead to the splitting of both NMR signal into various peaks with a determined spacing, which is defined as the

coupling constant (J) [13,14]. Typically, the coupling is observed in vicinal hydrogens, i.e. in hydrogens on adjacent carbons and can lead to the appearance of singlets, duplets, triplets, quartets or multiplets, which depends on the number and spin orientation of neighbouring nuclei as it will lead to frequency shifts to lower or higher fields [14]. Moreover, a major advantage in structural characterization is that spin-spin coupling is independent on the applied magnetic field as opposed to the chemical shift [14].

2.1.5. X-ray diffraction

The X-ray diffraction (XRD) technique is based on the constructive interference of incident X-rays over a crystalline structure owing to the diffraction caused by the atoms in the crystal [15,16]. The required equipment is an X-ray diffractometer which contains an X-ray tube, a sample holder and an X-ray detector. Electrons are produced by heating a filament in a cathode ray tube, which are then accelerated under the action of a voltage difference. The electrons impact onto a metal produces the required energy for the electrons to be dislodged from the atoms inner shells, where the incomplete electronic level is then filled by higher electronic level electrons, which is accompanied by the emission of X-ray photons. The X-ray wavelengths are characteristic of each metal, where copper is the most commonly used material, which are collimated and directed to the sample to be analysed [16]. The regularity of the atomic distribution in the crystal lattice produces diffracted X-rays that cancel each other, though in certain direction a constructive interference might occur [15], if the conditions satisfy the Bragg law:

$$n\lambda = 2d \sin \theta \quad (2.30)$$

where n is an integer number that denotes the reflection order, λ is the electromagnetic radiation wavelength, d is the distance between atomic planes and θ is the angle formed by the diffracted and incident radiation. The diffracted X-rays are then detected along 2θ angles, processed and counted [15]. Therefore, the collection of all possible diffracted X-rays allows the determination of the possible atomic plane distances and which mineral is being analysed by comparison with other reference patterns, as each material has its own characteristic d . Hereby, the non-destructive property of XRD has contributed for the technique application in a wide variety of studies encompassing structural properties, crystalline phases, crystallographic orientation and texture, crystal dimensions, crystallinity and perfection [15].

2.1.6. Circular dichroism

Electromagnetic radiation can be linearly or circularly polarized according to the direction defined by the electric field vector during the wave propagation. In a linearly polarized light the photons oscillate in a straight line along the same plane, while in circularly polarized light the electric field vector traces a helix along its propagation (figure 2.13) [17]. Hereby, a linearly polarized electromagnetic wave propagating in the z direction can be divided on its x and y axis projections oscillating in phase, while in the circularly polarized light the two components oscillate out of phase. If the phase difference (δ) occurs in $0 < \delta < \pi$ range, the wave rotates clockwise as it propagates away from the observer and anticlockwise when $\pi < \delta < 2\pi$, where $\delta = \frac{\pi}{2}$ is said to be circularly polarized [13].

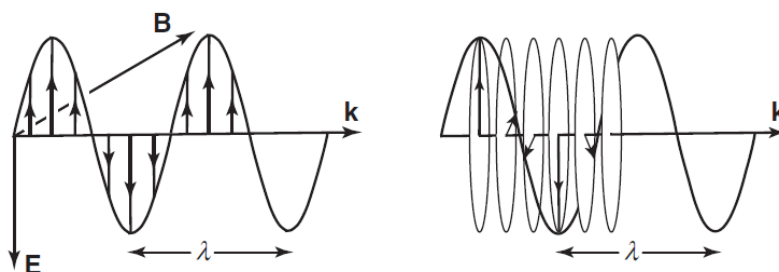


Figure 2.13. Schematic representation of (left) linearly and (right) circularly polarized electric wave vector. Adapted from [17].

The interaction of circularly polarized light with a molecule will result in the electronic transition from the lowest vibrational level of the ground-state to the various vibrational levels in the first excited-state if the molecule is optically active, i.e. the magnetic transition dipole moment vector has a component parallel to the electric transition dipole moment vector [13,18]. Here, the electric transition dipole moment vector can be represented as the linear displacement of charge induced in the transition, while the magnetic transition dipole moment vector is associated to the circular motion of charge [13]. Thus, for a molecule to be optically active, the chromophore must contain an asymmetric (chiral) environment or display an induced chirality owing to three-dimensional bonding structures, such as the α -helix and β -sheet conformations in peptide chains [19-21]. A consequence of the interaction of circularly polarized light with a chiral molecule is that left- and right-polarized will be absorbed differently, which leads to the definition of circular dichroism as the absorbance difference of left (A_L) and right (A_R) circularly polarized light [13,17]:

$$\Delta A = A_L - A_R = \Delta \epsilon bc \quad (2.31)$$

where $\Delta\epsilon$ is the decadic molar extinction coefficient variation. For historical reasons, the circular dichroism spectrum is represented in units of ellipticity (θ) versus λ that can be converted through the equation:

$$\theta(\text{degrees}) = \frac{180 (\ln 10) \Delta A}{4\pi} \equiv 32.982 \Delta A \quad (2.32)$$

The required equipment is a spectropolarimeter consisting on a light source, commonly a xenon arc lamp, where the emitted radiation is polarized and collimated through a series of prisms, mirrors and slits (figure 2.14). The linearly polarized light is then focused by a lens and passed through a filter comprising a series of tilted quartz plates that precede a photoelastic modulator made of a voltage-driven piezoelectric crystal coupled to a piece of isotropic material (fused quartz). The subjection to an alternating electric field induces structural changes that produce acoustically resonant changes of the crystal optical properties, which lead to the plate transmitting alternating left and right circularly polarised light with the frequency of the driving voltage [17,18,22]. The circularly polarized light is then passed through the sample compartment and detected by the photomultiplier [17,18,22].

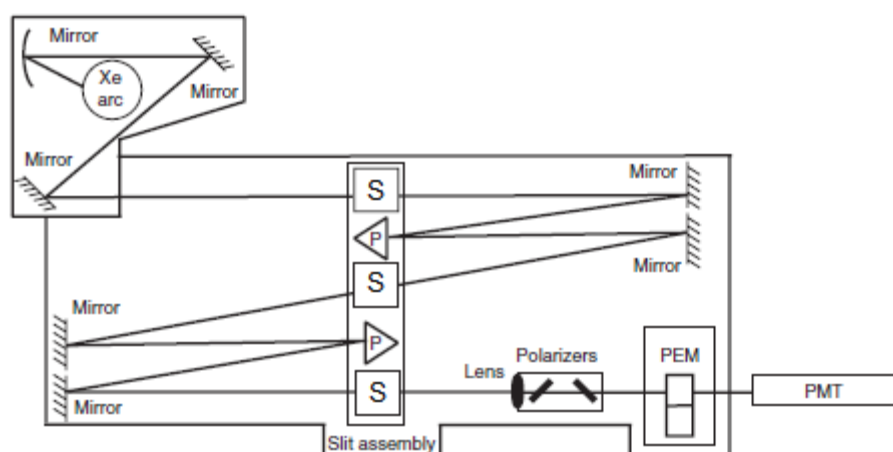


Figure 2.14. Schematic representation of a spectropolarimeter. Legend: S: slit; P: Prism; PEM: photoelastic modulator; PMT: photomultiplier. Adapted from [17].

2.2. Mass spectrometry

Mass spectrometry consists on the formation, separation and detection of ions according to its mass and charge [14]. When an ion with charge q is accelerated to a velocity v into a homogeneous magnetic field perpendicular to its direction, it is subjected to a perpendicular force (Lorentz force) [13]:

$$F = q(E + v \times B) \quad (2.33)$$

where E is the electric field. If the electric field is absent, the magnetic force will balance the centrifugal force leading the particle into a circular trajectory of radius r , thus allowing the separation of ions accelerated by an applied voltage V , according to its mass-to-charge ratio:

$$\frac{m}{q} = \frac{B^2 r^2}{2V} \quad (2.34)$$

Owing to the high ion reactivity, the formation and manipulation is conducted under high vacuum, being produced in an ion source such as an electrospray, matrix assisted laser desorption, single photon, electric or chemical ionization source [23]. The molecular ions are hereafter focused by ion optics and transferred through the mass analyser region, where ions are sorted by the mass-to-charge ratio either by using a static or dynamic magnetic/electric field, but all operating according to the Lorentz force and Newton's second law of motion [24]. The sorted ions are sent to a detector and the result is translated into a mass spectrum of intensity *versus* mass-to-charge ratio, where the intensity is proportional to the abundance of each ion. A general scheme of the mass spectrometer components is represented in figure 2.15.

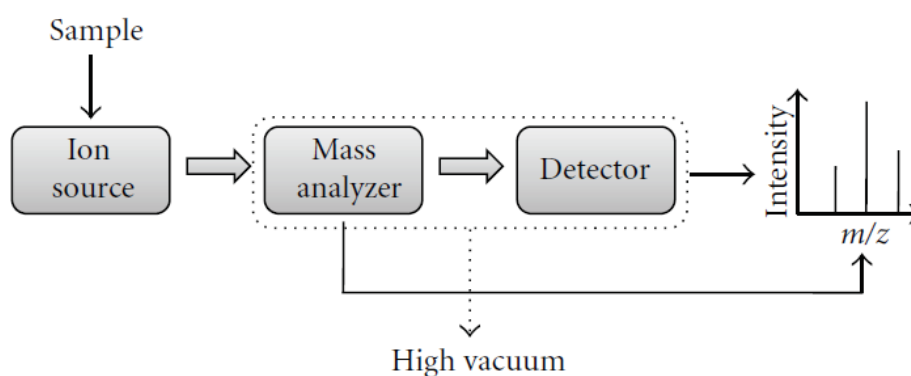


Figure 2.15. General scheme of the basic mass spectrometer components. Reprinted from [24].

2.3. Transmission electron microscopy

Electron microscopy has offered important advances on the development of new materials, as it can deal with a vast variety of specimens such as asymmetric particles, two-dimensional crystals or cells [13,25]. Images are a result of the interaction between electrons and the atoms in the specimen, similarly to the interaction of photons with a specimen in optical microscopy. Considering that the energy of an electron is a feature of its acceleration under an electric potential difference [13]:

$$eV = \frac{1}{2} \frac{p^2}{m} \quad (2.35)$$

where p is the momentum, m and e are the electron mass and charge, respectively, then the wavelength can be expressed as a function of the applied electric potential difference according to the equation [13]:

$$\lambda = \frac{h}{\sqrt{2meV}} \quad (2.36)$$

Therefore, electrons can attain the desired wavelength by varying the electric field potential, which are focused and directed to a specimen by electromagnetic lenses. As the minimum separation that can be resolved is proportional to the wavelength and inversely proportional to resolution, an increase of the electrons energy will increase the resolution.

In transmission electron microscopy (TEM), the image is formed owing to the interference between the electrons that passed through the sample and those that were not affected by it, while in scanning electron microscopy (SEM) the signal results from secondary electrons emitted by the sample [13].

Electrons can be generated by a heated tungsten filament at high vacuum that are accelerated at 100-400 keV through the electric potential difference between an anode and cathode. The accelerated electron beam is concentrated by a condenser lens and focused on the specimen by the objective lenses. The transmitted electrons pass an aperture (annular metallic plates) to exclude scattered electrons, positioned by projector lenses and strike an imaging device such as a fluorescent material coated screen or detected by a sensor such as a charge-coupled device (CCD), and the intensity is translated into an image [13].

2.4. Rheology

Rheology is the study of a materials flow and deformation as a function of force, time and spatial orientation when a force is applied [26,27]. When a force is applied over an area on a material (stress), a deformation (strain) is induced. In solid materials, the strain is defined as the change in dimensions relative to the original dimensions, which can be due to an applied tensile or shear stress. Hereby, an elastic modulus (G) is defined:

$$G = \frac{\tau}{\gamma} \quad (2.37)$$

where τ is the applied force per area and γ represents the strain (deformation or displacement). In fluids an applied stress changes the rate of strain with time rather than the amount of strain, i.e. the viscosity (η):

$$\eta = \frac{\tau}{\dot{\gamma}} \quad (2.38)$$

where $\dot{\gamma}$ is the shear strain rate. Fluids can be further classified as Newtonian or non-Newtonian fluids, according to whether viscosity is constant or it varies with an applied force or deformation. Hereby, the elastic modulus indicates how much a material deforms elastically when a force is applied, while viscosity indicates how fast the material flows as a response to it [26].

However, all materials can behave as solids or liquids (viscoelastic) depending upon the timescale, which leads to the dependence on both the elastic strain γ and viscous strain rate $\dot{\gamma}$ [27]. Therefore, to differentiate an elastic and viscous material, measurements are performed by an oscillatory deformation at different frequencies [26]. Such measurements are taken at small deformations (small amplitude oscillatory shear) to carry the experiment in the viscoelastic region, i.e. where the properties are independent of the imposed stress or strain [28]. If a stress or strain sinusoidal oscillation is applied in a sample, the material is elastic when the applied and measured waves are in-phase and viscous when out-of-phase by 90° , while the viscoelastic materials phase (δ) varies between 0° and 90° . Thus, a complex modulus can be defined as:

$$G^* = G' + iG'' \quad (2.39)$$

where the in-phase real component is defined as the storage modulus (G'), that measures the deformation stored energy (elastic behaviour) during shear process, while the out-of-phase imaginary component is the loss modulus (G'') and represents the dissipated energy as heat (viscous behaviour) [28].

The loss factor, $\tan \delta = \frac{G''}{G'}$ is a critical property of the hydrogels, as it indicates if they have a more viscous liquid ($\tan \delta > 1$) or an elastic solid ($\tan \delta < 1$) behaviour [28]. Moreover, the measurement of the moduli as a function of time allows following hydrogelation, while as a function of frequency indicates the behaviour of the hydrogel at different timescales [28].

2.5. Computational studies

2.5.1. Quantum chemical computation of molecular properties

Nowadays, *ab initio* computational methods have contributed to the study of various molecular properties such as dipolar moments, ionization energy and electron affinity, the molecular structure, photochemical properties and chemical reactions. Such is attained by the computational solution of the Schrödinger equation, given the position of a collection of atomic nuclei and the total number of electrons in the system [29]. One approximation in the computational methods is the Born-Oppenheimer approximation, which states that, as the motion of electrons is much faster than that of the nuclei, then the latter can be assumed to be fixed, thus solving the Schrödinger equation for fixed positions of the nuclei [30].

One of the most commonly used methods is the density functional theory method (DFT) that determines the ground-state electronic energy through the electronic density, which is an alternative to the Hartree-Fock self-consistent field method (HF-SCF) that treats the movement of an electron independently of the position of other electrons [30,31]. The neglect of the influence of Coulombic interactions on the repulsion of electrons (electron correlation) causes electrons to be closer in the HF-SCF than in DFT method [30].

The problem associated with the DFT method is the exchange-correlation functional not being known, which requires the use of approximations such as the local-density approximation (LDA), where the energy depends on the density at the point where the functional is evaluated, and the generalized gradient approximation (GGA) that incorporates a dependence on density gradient [31]. A common approximation is the B3LYP hybrid method that combines GGA with exact Hartree-Fock exchange [31].

The computational cost can be reduced with the use of the ONIOM approach (called Our own N-layered Integrated molecular Orbital and molecular Mechanics) where an entire “real” system can be divided in three systems (“model”, “intermediate” and “real”) and three levels of theory (“low”, “medium” and “high”) [32]. For a two-layer system, the “real” system energy at the high level can be determined according to [32]:

$$E_{high,real} = E_{high,model} + E_{low,real} - E_{low,model} \quad (2.40)$$

Nevertheless, for all the above-mentioned methods, a basis set is required, which is a combination of several Gaussian functions with different bandwidths to approximate real atomic functions. For example, an s-type basis function is described as [30]:

$$\psi(r) = \sum_i^N d_i e^{-a_i f^2 r^2} \quad (2.41)$$

where N is the number of Gaussian functions, d_i represents the weight of each Gaussian function to the basis function, a_i defines the width of the function, f is a scale factor and r is the distance from the nucleus [30]. The minimal basis set is the STO-3G that describes all orbitals with three Gaussian functions, while a split-valence basis set uses a combination of functions for the core orbitals different from the valence orbitals. An example is the 6-31G basis set, where six Gaussian functions are applied on the 1s orbitals, while two sets of Gaussian functions are applied to the valence orbitals [30]. A polarization function can be added, which consists on adding a function corresponding to higher angular momentum than those that are occupied, i.e. adding a d function to second row elements (6-31G(d)) and a p function to hydrogen atoms (6-31G(d,p)) [30]. Other possibility is the addition of diffuse functions to consider the electron density far from the nuclei in anions and atoms with non-bonding electrons, which can be added to second row elements (6-31+G) and to hydrogen atoms (6-31++G) [30].

2.5.2. Molecular docking

Molecular docking consists on the computational simulation of the physical processes involved in a binding event, the prediction of the molecular orientation in a binding site and the affinity between two molecules, which is attained by sampling conformations of a ligand in the target site and its ranking by a scoring function [33]. Sampling algorithms focus on finding the conformation that interacts most favourably with a receptor and including matching algorithms, and incremental construction, stochastic and molecular dynamics methods [33,34]. From the mentioned methods, the genetic algorithm (a stochastic method) has been commonly used for the ligand-protein docking, where the “genes” (degrees of freedom of the ligand encoded as binary strings) that make up the “chromosome” (the ligand’s pose) suffer “mutation” (random changes) and “crossover” (exchange genes between two chromosomes) that will result in a new ligand structure [33]. The chromosomes are then evaluated by a scoring function and the ones that survive will be used for the next generation. In *AutoDock* software, the genetic algorithm is further improved into a “Lamarckian” algorithm, where an energy minimization is performed after the

genotypic changes [34]. In this algorithm, “phenotypic” changes determined by the energy minimization of the genes are then mapped back onto the genes and the survivors pass to the next generation [34,35].

The scoring function evaluates which poses are correct, i.e. it estimates the binding affinity between the ligand and protein and scores the poses according to the binding energy through a scoring function that can be force-field-based, empirical or knowledge-based [33]. The force-field-based scoring functions consist on the use of a molecular mechanics force-field and the calculation of the sum of non-bonding interactions, while the empirical-based scoring decomposes the binding energy in various energy components multiplied by empirically determined coefficients [33]. In the AutoDock software, a semi-empirical method is used, which incorporates an Amber type force-field accounting for dispersion/repulsion, hydrogen bonding, electrostatics and desolvation terms that are empirically parameterized by various ligand-protein complexes of known structure and inhibition constants [36].

2.6. Superconducting quantum interference device (SQUID)

SQUID magnetometers are presently the most sensitive instruments to measure magnetic moments of liquid and solid-state samples, capable of measuring magnetic moments in the order of 10^{-8} emu. These equipments consist on a closed superconducting (lack of electrical resistance) loop including one or two thin insulating layers to form Josephson junctions [37]. Its operation is based on the Josephson effect, i.e. the tunnelling of superconducting electrons across a Josephson junction. In the Josephson loop, an applied bias current split equally between each Josephson junction (figure 2.16) [38]. A magnetic flux inductively coupled to the SQUID will create screening currents that will increase the current in one junction and decrease in the other, which creates a voltage drop across the junction [38]. As the magnetic flux changes due to a magnetic material, a periodic voltage change occurs with a period equal to that of the flux quantum, thus translating the magnetic fluctuations into electronic signals, which are relayed to a monitoring device allowing the determination of the applied magnetic flux [38]. The sample is mounted in a straw and moved along the symmetry axis of pick-up coils (superconducting detection coils) in the presence of a magnetic field held constant [39]. The pick-up coils are in a configuration such that the coil is only sensible to the sample’s magnetic field strays. Thus, a change in the magnetic flux will induce a change on the coil’s persistent current, which produce a change of magnetic flux on the input coil close to the SQUID sensor and, consequently, its output voltage [39].

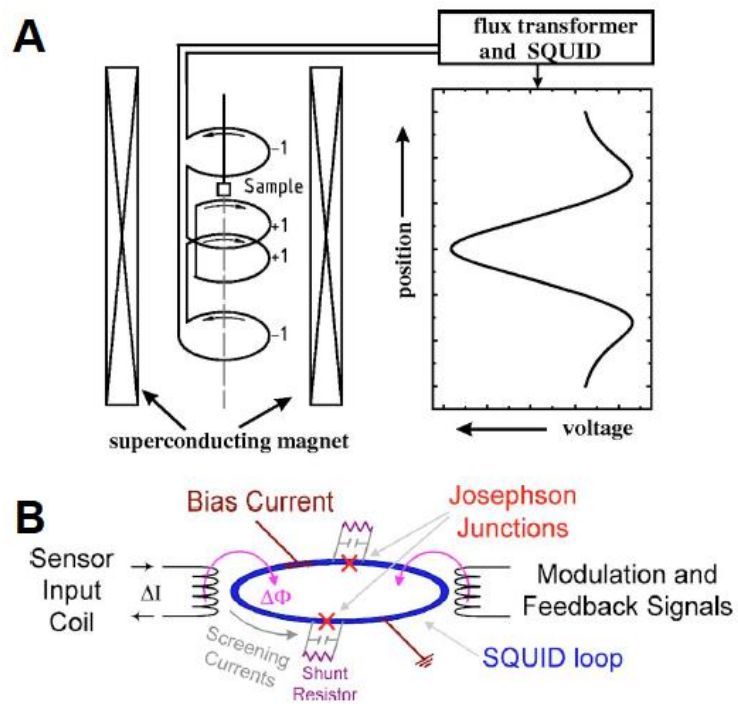


Figure 2.16. Schematic representation of (A) the pick-up coil and a theoretical signal in a SQUID magnetometer, and (B) the dual junction of the SQUID loop. The shunt resistors prevent the I-V hysteresis curve. Adapted from [38] and [39].

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Chapter 3 - Experimental section

3.1. Synthesis of dehydrodipeptide hydrogelators

3.1.1. Hydrogelator (S)-2-(6-methoxynaphthalen-2-yl)-(S)-methionyldehydraphenylalanine (Npx-L-Met-Z-ΔPhe-OH)

Synthesis of *tert*-butoxycarbonylmethionine [Boc-L-Met-OH (3)]: A solution of di-*tert*-butyl dicarbonate (2.35 g, 0.011 mol, 1 equiv) in 3.75 mL of *tert*-buty alcohol was added dropwise at rt to a stirring solution of L-methionine (1.58 g, 0.011 mol) in NaOH 1 M (10.6 mL, 1.1 equiv). A white precipitate was formed, and the resulted mixture was allowed to stir at rt overnight. The solution was acidified to pH 1-1.5 with a solution KHSO₄ 1 M. The turbid reaction mixture was extracted with ethyl acetate (2 x 20 mL), dried over magnesium sulfate anhydrous, filtered, and the solvent was removed under reduced pressure. Compound **3** was isolated as an oil (2.02 g; 81 %). This procedure was adapted from the reference [1].

¹H NMR (400 MHz, DMSO) δ: 1.37 (9H, s, 3 x CH₃ Boc); 1.79-1.87 (2H, m, β-CH₂ Met), 2.02 (3H, s, SCH₃ Met); 2.44-2.50 (2H, m, γ-CH₂ Met), 3.96-4.02 (1H, m, α-CH Met), 7.11 (1H, d *J* = 8 Hz, NH Met), 12.5 (1H, br s, CO₂H Met).

Synthesis of the methyl ester of β-hydroxyphenylalanine [H-D,L-Phe(β-OH)-OMe (4)]: The β-hydroxyphenylalanine [H-D,L-Phe(β-OH)-OH] (25 mmol, 4.52 g) was added to 40 mL of methanol in an ice bath. Thionyl chloride (3.4 equiv) was added dropwise and the reaction mixture was left stirring for 4 h at 40 °C. The solvent was removed under reduced pressure and ethyl ether was added. The process was repeated until a white solid of compound **4** was formed (98%, 5.68 g). This procedure was adapted from the reference [2].

¹H NMR (400 MHz, DMSO) δ: 3.59 (3H, s, OCH₃); 4.13-4.14 [1H, d, α-CH Phe(β-OH)]; 4.99-5.02 [1H, t, β-CH Phe(β-OH)]; 6.56 [1H, d (*J* = 4.4 Hz), OH Phe(β-OH)]; 7.31-7.38 [5H, m, ArH Phe(β-OH)]; 8.48 [3H, s, NH₃⁺ Phe(β-OH)].

Synthesis of the methyl ester of *N*-*tert*-butoxycarbonyl-L-methionyl-β-hydroxyphenylalanine [Boc-L-Met-D,L-Phe(β-OH)-OMe (5)]: Boc-L-Met-OH (2.02 g, 8.10 mmol) was dissolved in acetonitrile (20 mL) and put in an ice bath. HOBt (1.00 equiv, 1.24 g, 8.10 mmol), DCC (1.00 equiv, 1.67 g, 8.10 mmol), H-D,L-Phe(β-OH)-OMe (1.00 equiv, 1.88 g, 8.10 mmol), and triethylamine (2.00 equiv, 2.25 mL, 16.21 mmol) were added with 2 min between each addition. The mixture was left stirring at rt overnight. The urea was

filtered, and the solvent removed under reduced pressure. Acetone was added, and the mixture was stored in the freezer for 2 h. The urea was filter again. Evaporation at reduced pressure gave a residue that was partitioned between ethyl acetate (50 mL) and KHSO₄ (50 mL, 1M). The organic phase was thoroughly washed with KHSO₄ (1M, 2 x 50 mL), NaHCO₃ (1M, 2 x 50 mL) and brine (3 x 50 mL) and dried with MgSO₄. Removal of the solvent afforded compound **5** as a diastereomeric mixture (2.12 g, 84.2 %).

¹H NMR (400 MHz, DMSO) δ : 1.32-1.38 (9H, s, 3 x CH₃ Boc); 1.60-1.65 (2H, m, β -CH₂ Met); 2.17-2.26 (2H, m, γ -CH₂ Met); 2.49 (3H, s, SCH₃ Met); 3.64 (3H, s, OCH₃), 4.54-4.58 (1H, m, α -CH Met); 5.10 [1H, s, OH Phe(β -OH)]; 5.13-5.15 [1H, m, β -CH Phe(β -OH)]; 5.93-5.96 [1H, s, α -CH Phe(β -OH)]; 6.90-6.92 and 7.04-7.06 (1H, dd (J = 8.4 Hz), NH Met); 7.19-7.38 [5H, m, ArH Phe(β -OH)]; 7.78-7.80 and 7.92-7.94 (H, dd (J = 8.8 Hz and 9.2 Hz), NH Phe(β -OH)).

Synthesis of the methyl ester of *N*-*tert*-butoxycarbonyl-L-methionyl-Zdehydrophenylalanine [Boc-L-Met-Z- Δ Phe-OMe (6**)]:** To a solution of compound **5** in dry acetonitrile (10 mL, 1 M) DMAP (0.1 equiv, 0.082 g, 0.68 mmol) and Boc₂O (1 equiv, 1.49 g, 6.83 mmol) were added under rapid stirring at rt. The mixture was monitored by ¹H NMR until all reactant was consumed. *N,N,N',N'*-tetramethylguanidine (2 % in volume, 0.2 mL) was added under continued stirring. The mixture was left stirring at rt and monitored by ¹H NMR until all reactant was consumed. Evaporation at reduced pressure gave a residue that was partitioned between ethyl acetate (50 mL) and KHSO₄ (1 M, 30 mL), washed with KHSO₄ (1 M, 2 x 30 mL), NaHCO₃ (1 M, 3 x 30 mL), water (1 x 30 mL) and brine (2 x 30 mL) and dried with MgSO₄. Removal of the solvent afforded compound **6** (1.67 g, 60%).

¹H NMR (400 MHz, DMSO) δ : 1.38 (9H, s, CH₃ Boc); 1.85-1.92 (2H, m, β -CH₂ Met), 1.91-1.94 (2H, m, γ -CH₂ Met); 2.07 (3H, s, CH₃ Met); 3.69 (3H, s, OCH₃ Δ Phe); 4.14-4.16 (1H, m, α -CH Met); 7.11 (1H, d (J = 7.6 Hz), NH Met); 7.25 (1H, s, β -CH Δ Phe); 7.31-7.38 (3H, m, ArH Δ Phe), 7.70 (2H, d (J = 3.6 Hz), ArH Δ Phe); 9.64 (1H, s, NH Δ Phe).

Synthesis of the methyl ester of L,D-methionyl-Zdehydrophenylalanine [H-D,L-Met-Z Δ Phe-OMe,TFA (7**)]:** TFA (3 mL mmol⁻¹) was added to Boc-L-Met-Z Δ Phe-OMe (1.65 g, 4.04 mmol), and the mixture was left stirring 4 h at rt. The solvent was removed under pressure, ethyl ether was added and stored in the freezer for 1 h. The solvent was removed under reduced pressure. Precipitation from ethyl ether afforded compound **7** as a white solid (1.63 g, 95.4 %).

^1H NMR (400 MHz, CDCl_3) δ : 1.38 (3H, s, CH_3 Met); 1.59-1.62 (2H, m, $\beta\text{-CH}_2$ Met); 1.74-1.83 (2H, m, $\gamma\text{-CH}_2$ Met); 3.07-3.14 (1H, m, $\alpha\text{-CH}$ Met); 3.92 (3H, s, OCH_3 ΔPhe); 6.92 (1H, s, $\beta\text{-CH}$ ΔPhe); 7.11-7.16 (1H, m, ArH ΔPhe), 7.37-7.45 (2H, m, ArH ΔPhe), 7.69-7.72 (2H, m, ArH ΔPhe).

Synthesis of the methyl ester of *N*-naproxen-L-methionyl-*Z*-dehydrophenylalanine [Npx-L-Met-*Z* Δ Phe-OMe (8)]: Compound **7** was dissolved in dry DCM (5 mL mmol $^{-1}$) in an ice bath. Triethylamine (3 equiv; 0.42 mL) was added, followed by (S)-(+)-naproxen (1 equiv, 0.25 g), being left stirring overnight in an ice bath. A white precipitate was formed. The mixture was filtered and the solid was identified as compound **8** (0.15 g, 29 %). Alternatively, compound **7** was dissolved in DCM (5 mL mmol $^{-1}$) in an ice bath and HOBt (1.1 equiv, 0.53 g, 3.16 mmol), DCC (1.1 equiv, 0.72 g, 3.16 mmol), naproxen (1.1 equiv, 0.80 g, 3.16 mmol), and triethylamine (3.00 equiv, 1.32 mL, 9.49 mmol) were added with 2 min between each addition. The mixture was left stirring at rt overnight. The urea was filtered, and the solvent removed under reduced pressure. Acetone was added, and the mixture was stored in the freezer for 1 h. The precipitate was identified as compound **8** (0.61 g, 36.9 %).

^1H NMR (400 MHz, CDCl_3) δ : 1.61 (3H, d (J = 7.2 Hz), CH_3 Npx); 1.95-2.10 (2H, m, $\beta\text{-CH}_2$ Met), 2.00 (3H, s, CH_3 Met); 2.53-2.56 (2H, m, $\gamma\text{-CH}_2$ Met); 3.72-3.79 (1H, m, CH Npx); 3.74 (1H, s, OCH_3 ΔPhe); 3.91 (3H, s, OCH_3 Npx); 4.77 (1H, q (J = 6.4 Hz), $\alpha\text{-CH}$ Met); 6.47 (1H, d (J = 6.8 Hz), NH Met); 7.08 (1H, s, $\beta\text{-CH}$ ΔPhe); 7.12-7.15 (1H, m, ArH); 7.25-7.42 (7H, m, ArH); 7.63-7.66 (3H, m, ArH); 7.85 (1H, s, NH ΔPhe).

^{13}C NMR (100.6 MHz, CDCl_3) δ : 15.03 (SCH_3); 18.29 (CH_3 Npx); 29.68 ($\beta\text{-CH}_2$ Met); 29.97 ($\gamma\text{-CH}_2$ Met); 46.96 ($\alpha\text{-CH}$ Npx); 52.56 (OCH_3 ΔPhe); 52.60 ($\alpha\text{-CH}$ Met); 55.31 (OCH_3 Npx); 105.61 (CH Npx); 119.19 (CH Npx); 123.53 (C ΔPhe); 125.94 (CH Npx); 126.19 (CH Npx); 127.71 (CH Npx); 128.59 (CH ΔPhe); 128.98 (C Npx); 129.24 (CH Npx); 129.59 (CH ΔPhe); 129.70 (CH ΔPhe); 133.29 (C ΔPhe); 133.36 (CH ΔPhe); 133.82 (C Npx); 135.61 (C Npx); 157.78 (C Npx); 165.09 (C=O ΔPhe); 169.83 (C=O Met); 174.92 (C=O Npx).

Synthesis of the *N*-naproxen-L-methionyl-*Z*-dehydrophenylalanine [Npx-L-Met-*Z* Δ Phe-OH (1)]: The (S)-(+)-naproxen-dehydrideptide (0.120 g, 0.23 mmol) was dissolved in acetone (10 mL mmol $^{-1}$) and a solution of NaOH 1 M (6 equiv) was added. The reaction was followed by TLC until no starting material was detected. The organic solvent was removed under reduced pressure, and the reaction mixture was acidified to pH 2-3 with KHSO_4 (1 M). The solid was filtered and washed with ethyl ether. The solid was identified as Npx-L-Met-*Z* Δ Phe-OH, **1** (0.115 g, 98.9 %).

¹H NMR (400 MHz, DMSO) δ : 1.42 (3H, d (J = 7.2 Hz), CH₃ Npx); 1.83-2.05 (2H, m, CH₂ Met), 2.05 (3H, s, CH₃ Met); 2.29-2.32 (2H, m, CH₂ Met); 3.83-3.85 (1H, m, CH Npx), 3.83 (3H, s, OCH₃ Npx); 4.44-4.54 (1H, m, α -CH Met); 7.08-8.26 (11H, m, ArH); 8.27 (1H, d = 8 Hz, NH Met); 9.52 (1H, s, NH Δ Phe).

¹³C NMR (100.6 MHz, DMSO) δ : 14.67 (SCH₃); 19.03 (CH₃ Npx); 29.41 (β -CH₂ Met); 31.76 (γ -CH₂ Met); 44.61 (CH Npx); 52.01 (α -CH Met); 55.16 (OCH₃ Npx); 105.69 (CH Npx); 118.45 (CH Npx); 125.40 (CH Npx); 126.39 (C Δ Phe); 126.50 (CH Npx); 126.66 (CH Npx); 128.37 (C Npx); 128.39 (CH Δ Phe); 129.09 (CH Npx); 129.12 (CH Δ Phe); 129.92 (CH Δ Phe); 132.14 (CH Δ Phe); 133.11 (C Npx); 133.43 (C Δ Phe); 137.21 (C Npx); 156.96 (C Npx); 166.11 (C=O Δ Phe); 170.98 (C=O Met); 173.65 (C=O Npx). HR-MS (ESI) m/z . [M + H]⁺ calcd for C₂₈H₃₁N₂O₅S⁺ 507.19; found, 507.19. Mp: 189-192 °C.

3.1.2. Hydrogelator (S)-2-(6-methoxynaphthalen-2-yl)-(S)-lysyldehydrophenylalanine (Npx-L-Lys-Z Δ Phe-OH)

Synthesis of the methyl ester of *N*-fluorenylmethyloxycarbonyl-L-lysyl(*N*-tert-butoxycarbonyl)- β -hydroxyphenylalanine [Fmoc-L-Lys(Boc)-D,L-Phe(β -OH)-OMe (**9**)]: Fmoc-L-Lys(Boc)-OH (0.94 g, 2 mmol) was dissolved in acetonitrile (20 mL) and put in an ice bath. HOBt (1.00 equiv, 0.31 g, 2 mmol), DCC (1.00 equiv, 0.41 g, 2 mmol), H-D,L-Phe(β -OH)-OMe (1.00 equiv, 0.46 g, 2 mmol), and triethylamine (4.00 equiv, 0.56 mL, 8 mmol) were added with 2 min between each addition. The mixture was left stirring at rt overnight. The urea was filtered, and the solvent removed under reduced pressure. Acetone was added, and the mixture was stored in the freezer for 2 h. The urea was filter again. Evaporation at reduced pressure gave a residue that was partitioned between ethyl acetate (50 mL) and KHSO₄ (50 mL, 1M). The organic phase was thoroughly washed with KHSO₄ (1M, 2 x 50 mL), NaHCO₃ (1M, 2 x 50 mL) and brine (3 x 50 mL) and dried with MgSO₄. Removal of the solvent afforded compound **9** as a diastereomeric mixture (1.20 g, 93.1 %).

¹H NMR (400 MHz, CDCl₃) δ : 1.11-1.23 (2H, m, γ -CH₂ Lys); 1.28-1.37 (2H, m, δ -CH₂ Lys); 1.42 (9H, s, CH₃ Boc); 1.55-1.52 (2H, m, β -CH₂ Lys); 2.98-3.08 (2H, m, ϵ -CH₂ Lys); 3.73 [3H, s, OCH₃ Phe(β -OH)]; 4.19-4.39 [2H, m, α -CH Lys and Phe(β -OH)]; 4.60-4.72 [1H, ds, OH Phe(β -OH)]; 4.86-4.94 (1H, dd (J = 6 Hz), CH Fmoc); 5.32-5.35 (1H, dd (J = 2.4 Hz), β -CH); 5.41-5.58 (1H, dd J = 8 Hz), CH₂

Fmoc); 6.94-7.01 (1H, m, NH Lys); 7.10-7.20 (1H, m, NH Lys); 7.26-7.41 [10H, m, ArH and NH Phe(β -OH)]; 7.57-7.58 (2H, m, ArH Fmoc); 7.75-7.78 (2H, m, ArH Fmoc).

Synthesis of the methyl ester of *N*-naproxen-L-lysyl(*N*-tert-butoxycarbonyl)-*Z*-dehydrophenylalanine [Npx-L-Lys(Boc)-*Z* Δ Phe-OMe (10**)]:** To a solution of compound **9** in DMF (2 mL) piperidine (2 %, 0.5 mL) was added and left for 10 min under rapid stirring at rt. Evaporation at reduced pressure gave a residue that was partitioned between ethyl acetate (50 mL) and water (50 mL) and dried with MgSO₄. Removal of the solvent afforded H-D,L-Lys(Boc)-D,L-Phe(β -OH)-OMe and dibenzofulvene. H-D,L-Lys(Boc)-D,L-Phe(β -OH)-OMe was dissolved in dry DCM (5 mL mmol⁻¹) in an ice bath. Triethylamine (2 equiv, 0.52 mL) was added, followed by (S)-(+)-naproxen (1 equiv, 0.46 g), being left stirring overnight in an ice bath. The mixture was filtered. Evaporation at reduced pressure gave a residue that was partitioned between ethyl acetate (50 mL) and KHSO₄ (50 mL, 1M). The organic phase was thoroughly washed with KHSO₄ (1M, 2 x 50 mL), NaHCO₃ (1M, 2 x 50 mL) and brine (3 x 50 mL) and dried with MgSO₄. Removal of the solvent afforded Npx-L-Lys(Boc)-D,L-Phe(β -OH)-OMe and dibenzofulvene. To a solution of Npx-L-Lys(Boc)-D,L-Phe(β -OH)-OMe in dry acetonitrile (10 mL, 1 M) DMAP (0.1 equiv, 0.02 g, 0.19 mmol) and Boc₂O (1 equiv, 1.49 g, 1.86 mmol) were added under rapid stirring at rt. The mixture was monitored by ¹H NMR until all reactant was consumed. *N,N,N',N'*-tetramethylguanidine (2 % in volume, 0.2 mL) was added under continued stirring. The mixture was left stirring at rt and monitored by ¹H NMR until all reactant was consumed. Evaporation at reduced pressure gave a residue that was partitioned between ethyl acetate (50 mL) and KHSO₄ (1 M, 30 mL), washed with KHSO₄ (1 M, 2 x 30 mL), NaHCO₃ (1 M, 3 x 30 mL), water (1 x 30 mL) and brine (2 x 30 mL) and dried with MgSO₄. Removal of the solvent gave a white solid that was submitted to a column chromatography starting with 100 mL of petroleum ether, followed by 50 mL fractions of mixtures of diethyl ether: petroleum ether and fractions of ethyl acetate. Evaporation of diethyl ether: petroleum ether and ethyl acetate fractions afforded compound **10** (0.21 g, 18.37 %).

¹H NMR (400 MHz, DMSO) δ : 1.34-1.43 (10H, m, γ -CH₂ Lys, δ -CH₂ Lys, CH₃ Npx and CH₃ Boc); 1.57-1.74 (2H, m, β -CH₂ Lys); 2.88-2.91 (2H, t (J = 6.4 Hz), ϵ -CH₂ Lys); 3.62 (3H, s, OCH₃ Δ Phe); 3.83 (3H, s, OCH₃ Npx); 3.86 (1H, q (J = 6.4 Hz), α -CH Npx); 4.44 (1H, q (J = 8 Hz), α -CH Lys); 6.76 (1H, t (J = 5.6 Hz), NH Lys); 7.08-7.11 (1H, dd (J = 2.4 Hz), ArH Npx); 7.17-7.20 (3H, m, ArH Δ Phe), 7.19 (1H, s, β -CH Δ Phe); 7.23-7.24 (1H, d (J = 2.4 Hz), ArH Npx); 7.44-7.47 (1H, dd (J = 2 Hz), ArH Npx); 7.54-7.57 (1H, m, ArH); 7.69 (4H, t (J = 6.8 Hz and 8.4 Hz), ArH); 8.20 (1H, d (J = 7.6 Hz), NH Lys); 9.66 (1H, s, NH Δ Phe).

Synthesis of the methyl ester of *N*-naproxen-L-lysine-*Z*-dehydrophenylalanine [Npx-L-Lys-*Z*ΔPhe-OMe (11)]: TFA (3 mL mmol⁻¹) was added to Npx-L-Lys(Boc)-*Z*ΔPhe-OMe (0.21 g, 0.34 mmol), and the mixture was left stirring 4 h at rt. The solvent was removed under pressure, ethyl ether was added and stored in the freezer for 1 h. The solvent was removed under reduced pressure. Precipitation from ethyl ether afforded compound **11** as a brown solid (0.18 g, 100 %).

¹H NMR (400 MHz, DMSO) δ: 1.37-1.44 (5H, m, γ-CH₂Lys and CH₃Npx); 1.55-1.58 (5H, m, δ-CH₂ and NH₃⁺ Lys); 1.74 (2H, m, β-CH₂ Lys); 2.76-2.78 (2H, m, ε-CH₂ Lys); 3.63 (3H, s, OCH₃ΔPhe); 3.84-3.88 (1H, m, α-CH Npx), 3.84 (3H, s, OCH₃Npx); 4.43-4.48 (1H, q (J = 7.6 Hz), α-CH Lys); 7.08-7.11 (1H, dd (J = 2.4 Hz), ArH Npx); 7.16-7.18 (3H, m, ArH ΔPhe); 7.22 (1H, s, β-CH ΔPhe); 7.23-7.24 (1H, d (J = 2.4 Hz), ArH Npx); 7.44-7.47 (1H, dd (J = 1.2 Hz), ArH Npx); 7.54-7.56 (1H, dd (J = 2.4 Hz), ArH); 7.67-7.71 (4H, m, ArH); 8.24-8.26 (1H, d (J = 8.4 Hz), NH Lys); 9.67 (1H, s, NH ΔPhe). HR-MS (ESI) *m/z*: [M + H]⁺ calcd for C₃₀H₃₆N₃O₅⁺ 518.26; found, 518.27.

Synthesis of *N*-naproxen-L-lysine-*Z*-dehydrophenylalanine [Npx-L-Lys-*Z*ΔPhe-OH (2)]: The (S)-(+)-naproxen-dehydrodipeptide (0.18 g, 0.23 mmol) was dissolved in 1,4-dioxane (10 mL mmol⁻¹) and solution of NaOH 1 M (2 equiv). The reaction was followed by TLC until no starting material was detected. The organic solvent was removed under reduced pressure, and the reaction mixture was acidified to pH 2-3 with KHSO₄ (1 M). Ethyl acetate was added, and an emulsion was formed. The isolated emulsion was dried and washed with ethyl ether. The solid was identified as Npx-L-Lys-*Z*ΔPhe-OH, **2** (0.11 g, 66.14 %).

¹H NMR (400 MHz, DMSO, 70 °C) δ: 1.41-1.43 (5H, m, γ-CH₂Lys and CH₃Npx), 1.55-1.75 (7H, d, δ-CH₂, NH₃⁺ and β-CH₂ Lys); 2.76 (2H, t (J = 4.2 Hz), ε-CH₂ Lys); 3.84-3.89 (1H, m, α-CH Npx), 3.84 (3H, s, OCH₃Npx); 4.45 (1H, q (J = 7.2 Hz), α-CH Lys); 7.05-7.06 (3H, m, ArH ΔPhe); 7.08-7.11 (1H, dd (J = 2.4 Hz), ArH Npx); 7.16 (1H, s, β-CH ΔPhe); 7.24 (1H, d (J = 2.8 Hz), ArH Npx); 7.42-7.47 (3H, m, ArH); 7.66-7.71 (3H, m, ArH); 8.19-8.21 (1H, d (J = 7.6 Hz), NH Lys); 9.34 (1H, s, NH ΔPhe), 12.63 (1H, brs, COOH).

¹³C NMR (100.6 MHz, DMSO) δ: 19.06 (CH₃ Npx); 21.81 (γ-CH₂ Lys); 26.51 (β-CH₂ Lys); 31.19 (δ-CH₂ Lys); 38.58 (ε-CH₂ Lys); 44.56 (CH Npx); 52.28 (α-CH Lys); 55.15 (OCH₃ Npx); 105.67 (CH Npx); 118.45 (CH Npx); 125.37 (CH Npx); 126.50 (CH Npx); 126.64 (CH Npx); 128.11 (C ΔPhe); 128.18 (CH ΔPhe); 128.37 (C Npx); 128.41 (CH ΔPhe); 129.08 (CH Npx); 129.63 (CH ΔPhe); 130.09 (CH ΔPhe); 133.11 (C Npx); 134.14 (C ΔPhe); 137.28 (C Npx); 156.95 (C Npx); 166.59 (C=O ΔPhe); 170.82 (C=O

Met); 173.48 (C=O Npx). HR-MS (ESI) m/z : $[M + H]^+$ calcd for $C_{29}H_{34}N_3O_5^+$ 504.24; found, 504.24. Mp: 218-220 °C.

3.2. Preparation of pH buffers

The pH buffer solutions in the pH range 2–10 were prepared from a sodium phosphate 0.1 M solution, and a mixed citric and boric acid solution at 0.05 M and 0.2 M, respectively, in ultrapure water (Milli-Q grade), for a final volume of 50 mL. The required volumes are presented in table 1, as described in [3]. The pH=11 and pH=12 buffers were prepared according to reference [4], by mixing a mixture of KCl 0.2 M (25 mL) and NaOH 0.2 M (6 mL or 66 mL) [4]. The pH was measured with a mini-pH meter NiCd-1 and the required adjustments were made using HCl or NaOH 1 M.

Table 3.1. Volumes required for the preparation of the buffer solution [3].

pH	Sodium phosphate (mL)	Citric/boric acid (mL)
2	1.25	48.75
3	6	44
4	11.25	38.375
5	16.5	33.5
6	20.5	29.5
7	25.25	24.75
8	28.75	21.25
9	32.75	17.25
10	36.5	13.5

3.3. Hydrogelators characterization

3.3.1. Spectroscopic measurements

Fluorescence measurements were carried out using a Fluorolog 3 spectrofluorimeter, equipped with double monochromators in both excitation and emission, Glan-Thompson polarizers and a temperature-controlled cuvette holder. Fluorescence emission spectra were corrected for the instrumental response of the system. The excitation of the hydrogelators was set at 290 nm (naproxen), and the emission spectrum was collected between 300 nm and 550 nm with a slit of 7 nm in both excitation and emission. Absorption spectra were recorded in a Shimadzu UV-3600 Plus UV-Vis-NIR spectrophotometer.

3.3.1.1. Self-assembly dependence on pH

An ethanolic solution of the hydrogelators was used to prepare aqueous solutions of the respective hydrogelators at a concentration of 0.2 μM and at the pH range 2–10. The emission spectra were obtained for each hydrogelator solution to evaluate the pH influence on the hydrogelators behaviour.

3.3.1.2. Critical gelation concentration

To determine the critical gelation concentration through fluorescence spectroscopy, aqueous solutions at a concentration range 0.2-0.8 wt% were prepared for each hydrogelator. The pH was chosen according to the results obtained in the self-assembly study of each hydrogelator at different pH values. For the hydrogelator Npx-L-Met-Z Δ Phe-OH, the solutions were prepared at pH=6. The hydrogelator Npx-L-Lys-Z Δ Phe-OH followed different protocols, thus in the first method the hydrogelator was solubilized in an organic solvent (DMSO, 2.5 v/v%) prior to addition of the pH=7 buffer, followed by homogenisation through sonication. The second method required solubilization of the hydrogelator at pH=13 and the hydrogel was formed at this pH using a heating-cooling cycle.

3.3.1.3. Critical aggregation concentration

The critical aggregation concentration was assessed through titration of the hydrogelator in the respective pH buffer. The concentration range to consider was determined by sequentially preparing 10x diluted solutions and the range was chosen according to where a fluorescence increase, or decrease, was observed.

3.3.1.4. Determination of fluorescence quantum yields

The determination of the hydrogelators fluorescence quantum yield, Φ_s , was carried out using the equation (standard method) [5-7],

$$\Phi_s = \frac{(A_r F_s n_s^2)}{(A_s F_r n_r^2)} \Phi_r \quad (3.1)$$

where A is the absorbance at the excitation wavelength, F the integrated emission area, n is the refraction index of the solvents, subscripts r and s , refer to reference and sample compound, respectively. The absorbance value at excitation wavelength was always less than 0.1, in order to avoid inner filter effects. L-Tryptophan in aqueous buffer solution (pH=7.2) was used as reference (Φ_r =0.14 at 25 °C) [8].

3.3.2. Computational assays

3.3.2.1. Molecular properties

The naproxen geometry was optimized with a 6-311+G(d,p) basis set at the DFT B3LYP level of theory, followed by energy calculation with a 6-311+G(d,p) basis set at the TD-SCF B3LYP level of theory. The optimized geometry was confirmed as a true energy minimum due to the lack of imaginary vibrational frequency values. The optimized geometries of the hydrogelators ground state were obtained from *ab initio* calculations, with the use of ONIOM method, setting the naproxen moiety for the high level with a 6-311+G(d,p) basis set at the DFT B3LYP level of theory and the peptide backbone for the low level at 3-21G basis set and HF B3LYP level of theory in gas phase. The energy calculation was taken at the 6-311+G(d,p) basis set at the TD-SCF DFT B3LYP level of theory for the naproxen moiety and 6-31G basis set at the TD-SCF DFT B3LYP level of theory for the peptide backbone. A lower basis set was used for the peptide backbone to attain a reasonable balance in the required processing time cost while preserving a good accuracy.

3.3.2.2. Docking studies in cyclooxygenase-1 and -2

The crystal structure of *Mus musculus* cyclooxygenase-2 (COX-2) expressed in *Spodoptera frugiperda* in complex with naproxen (PDB code: 3NT1) and *Ovis aries* cyclooxygenase-1 (COX-1) expressed in *Spodoptera frugiperda* in complex with flurbiprofen (PDB code: 3N8Z) were used as the protein receptor models. Docking of the receptor protein with the hydrogelators was performed using AutoDockTools-1.5.6 software Lamarckian Genetic Algorithm. The calculation was set up to 50 runs, 270 000 maximum number of generations, 2 500 000 maximum number of energy evaluations, 60x60x60 grid points for 3NT1 and 3N8Z with 0.375 Å spacing. The macromolecule was kept rigid and ligand molecules were flexible. The protocol was defined as adequate if the direct docking of the crystallographic molecule (naproxen and flurbiprofen) has a root-mean-square deviation lower than 2 Å. The lowest docked energy

conformation with the naproxen moiety in the active site was chosen as the best conformation. Visualization of the complex protein-ligand interactions was analysed with PyMOL software.

3.4. Magnetic/plasmonic nanoparticle synthesis

3.4.1. Manganese ferrite nanoparticles

Manganese ferrite nanoparticles were synthesized by the co-precipitation method in a solution with a total volume of 5 mL, as described by Rodrigues *et al.* [9]. Initially, a mixture of 500 μ L of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 0.5 M aqueous solution and 500 μ L of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ aqueous solution 1 M was prepared and added, drop by drop, to an aqueous solution with 612 μ L of NaOH 50% at 90 °C with constant magnetic stirring. After 2 h at 90 °C, nanoparticles were formed, and purification was carried out by repeated centrifugations, dispersion in deionized water, and drying at 100 °C.

3.4.2. Core/shell manganese ferrite/gold nanoparticles

The synthesis of gold-coated manganese ferrite nanoparticles was adapted from a previously reported method described in reference [10]. Thus, a 5 mL of MnFe_2O_4 (40 mg) nanoparticles aqueous dispersion were added to 25 mL of glycerol and left stirring until 200 °C, in a silicone oil bath. A solution of $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$ (2 mL, 0.04 M) was added dropwise and stirring was kept for 15 min. The mixture was washed with ethanol through centrifugation, followed by magnetic separation and left to dry at 100 °C.

3.4.3. Gold nanoparticles functionalized with 3-mercaptopropionic acid

The synthesis of gold nanoparticles functionalized with 3-mercaptopropionic acid (MPA) followed a procedure adapted from a previously reported method described in references [10,11]. 60 mg of gold(III) chloride hydrate, $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$, were dissolved in ethanol (50 mL), followed by the addition of 3-mercaptopropionic acid (3.5 mg) under vigorous stirring. A solution of NaBH_4 (50 mg) in NaOH 1 M (3 mL) was added dropwise and the mixture was left stirring for 30 min. A black/brown precipitate was formed, and purification was attained by centrifugation and washing with ethanol. The product was dried and stored in the freezer.

3.4.4. Gold-decorated manganese ferrite nanoparticles

The synthesis of gold-decorated manganese ferrite nanoparticles was adapted from a previously reported method in reference [10]. The MnFe_2O_4 nanoparticles (9 mg) were washed with NaOH 1M, dried and dispersed in DMSO (5 mL). Then, CDI (1,1'-carbonyldiimidazole) (63.36 mg) was added and the reaction was left for 2 h under vigorous stirring above 60 °C. The mixture was washed by repeated centrifugation and addition of DMSO. The nanoparticles were dispersed in DMSO, MPA-functionalized gold nanoparticles (35.7 mg) were added, and the reaction was left 1 h under vigorous stirring above 60 °C. The product of the reaction was obtained by magnetic separation. The nanoparticles were purified by repeated centrifugation and washed with DMSO. The gold-decorated nanoparticles were further functionalized with 2-aminoethanol (0.2 g) using the CDI (63.33 mg) coupling procedure described above. The product was magnetically separated and washed with water to remove the reagents and DMSO.

An alternative coupling method of MPA-gold nanoparticle to MnFe_2O_4 coupling method was proposed where the magnetic nanoparticles are mixed in DMSO, DMF or dioxane at 80-90 °C for 4 h or the required time to attain a translucent solution when a magnetic field is applied.

3.5. Photophysical hydrogel and magnetogel studies

3.5.1. Incorporation of nanoparticles into hydrogel matrixes

To evaluate the incorporation of core/shell and gold-decorated manganese nanoparticles into the hydrogels, emission spectra of the hydrogels with and without nanoparticles were obtained. The hydrogels were prepared for a final volume of 200 μL at 1 wt% (table 3.2). The nanoparticles were added for a final concentration of 0.1 wt% from a starting solution at 2 wt%. The required amount of the hydrogelator Npx-L-Lys-Z Δ Phe-OH in buffer was added and the mixture was stirred at 80 °C. The hydrogelator Npx-L-Met-Z Δ Phe-OH was dissolved at pH 9-10 by the addition of 2 v/v% NaOH 1 M and gelation was triggered with GdL (0.5 wt%) [2].

Table 3.2. Conditions used in the preparation of hydrogels/magnetogels from dehydrodipeptides Npx-L-Met-Z Δ Phe-OH and Npx-L-Lys-Z Δ Phe-OH for photophysical studies.

Hydrogelator	pH	wt%	GdL wt%
Npx-L-Met-Z Δ Phe-OH	6	1	0.5
Npx-L-Lys-Z Δ Phe-OH	13	1	-

The gelification of 150 μ L of each hydrogelator solution was carried out in a fluorescence microcuvette. The emission spectra were obtained between 300 nm and 550 nm, with excitation at 290 nm (naproxen), using 6 nm slit in both emission and excitation.

3.5.2. Incorporation of drugs into hydrogels and magnetogels

The hydrogels were prepared according to the conditions previously described in section §3.5.1 and curcumin was added for a final concentration of 2 μ M. From the hydrogelators solution, 200 μ L was transferred to a fluorescence microcuvette and left standing until the hydrogel/magnetogel was formed.

The drug incorporation was evaluated by the Föster resonance energy transfer (FRET) process between the hydrogel/magnetogel naproxen moiety and curcumin. The emission spectra were obtained through excitation of naproxen moiety at 290 nm or curcumin at 420 nm. In the FRET process, the naproxen fluorophore is the energy donor and curcumin is the energy acceptor.

3.5.3. Interaction of hydrogels and magnetogels with biomembrane models

Small unilamellar vesicles (SUVs) were used as biomembrane models. These were prepared from egg phosphatidylcholine and cholesterol at a 7:3 ratio, using the ethanolic injection technique [12]. Nile Red was incorporated into SUVs for a final concentration of 2 μ M. The required amount of cholesterol solution was evaporated with a stream of ultrapure nitrogen followed by the addition of the required volumes of ethanolic solutions of Nile Red and egg phosphatidylcholine. The mixture was added drop-by-drop to ultrapure water under vortex vigorous agitation. The SUVs solution was added to the hydrogels/magnetogels previously prepared into the microcuvettes in a 1:1 ratio. The interaction process was evaluated through FRET between curcumin and Nile Red, where curcumin is the energy donor and Nile Red is the energy acceptor.

3.5.4. Photothermia assays in magnetogels

Hydrogels containing curcumin (2 μM) were subjected to increasing temperatures to measure the dependence of fluorescence emission on temperature. Hydrogels/magnetogels incorporating curcumin were irradiated with a setup consisting on a Xenon arc lamp (200 W) and an optical fiber, using a Thorlabs FEL0600 long pass filter with cut-on wavelength at 600 nm to ensure no curcumin excitation. The detection was attained using a SPEX Fluorolog 2 spectrofluorimeter for 5 hours. At the fifth hour the irradiation was stopped, and fluorescence emission was measured one hour afterwards. The dependence of fluorescence emission on temperature was used to extrapolate on the local temperature created by excitation on plasmon band of the irradiated magnetogels.

3.5.5. Drug release assays

Hydrogels/magnetogels loaded with 20 μM curcumin were prepared. 100 μL were transferred to a microcuvette and left stabilizing overnight. A solution of biomembrane models (SUVs), prepared as previously described in §3.5.3, without Nile Red, was added (200 μL) to the top of the hydrogel/magnetogel. The curcumin accumulation into SUVs was monitored for 8 hours, with and without irradiation of the magnetogel.

The release of curcumin to pH=7 buffer followed a similar procedure as described for biomembrane models. Hydrogels/magnetogels loaded with 20 μM curcumin were prepared (200 μL) and left stabilizing overnight. The hydrogel/magnetogel was washed five times with 1mL of pH=7 buffer and water (1.5 mL) was added to the top of the hydrogel. Aliquots of 300 μL were taken, replaced with pH=7 buffer and the curcumin fluorescence emission was measured to determine the concentration at each time point.

3.6. Structural characterization

3.6.1. FTIR-ATR

Fourier Transform Infrared Spectroscopy (FTIR) measurements with Attenuated Total Reflection (ATR) were performed in a Bruker 66V spectrometer, with a resolution of 1 cm^{-1} .

3.6.2. Circular Dichroism

Circular Dichroism (CD) spectra were recorded at 20 °C on a spectropolarimeter Jasco model J-1500 spectropolarimeter (JASCO, Tokyo, Japan), at 25 °C, under a constant flow of nitrogen gas. Peptide hydrogelator 0.02 wt% solutions were loaded into 0.1 mm quartz cells. Spectra were acquired with 1 nm steps, 1 nm bandwidth and 1 second collection time per step, taking three averages. The obtained data was smoothed by an 11-point Savitsky-Golay filter, to remove random noise elements from the averaged spectra.

3.6.3. TEM

HR-TEM images were recorded using a Transmission Electron Microscope JEOL JEM 2010F operating at 200 kV coupled to an Electron Dispersive Spectroscopic analyser (EDS) at C.A.C.T.I (Centro de Apoio Científico e Tecnológico à Investigação), Vigo, Spain. The samples were prepared according to the uranyl acetate staining method. After preparation of the hydrogelators solutions, through prior dissolution at pH=12 followed by addition of GdL, a drop of each sample was placed onto a TEM 400 mesh copper grid with Formvar/Carbon (ref. S162-4 from Agar Scientific), held by tweezers and the excess solution was cleaned. A droplet of uranyl acetate was placed over the sample, the excess solution was cleaned, and the grid was left to dry. The processing of TEM images was performed using ImageJ software, which consisted in enhancing local contrast and adjusting brightness, followed by manual selection of particles and fibres.

3.6.4. X-Ray Diffraction

A conventional PAN'alytical X'Pert PRO diffractometer (at UME-UTAD) was used for X-ray diffraction (XRD) analyses, operating with Cu K α radiation, in a Bragg-Brentano configuration.

3.7. SQUID

Magnetic measurements were carried out at room temperature in a Superconducting Quantum Interference Device (SQUID) magnetometer (Quantum Design MPMS5XL), applying magnetic fields up to 7.0 T.

3.8. Rheology

The viscoelastic characterization of gels was performed with a stress-controlled rotational rheometer (MCR300, Anton Paar). Liquid samples were loaded into the Couette geometry of the rheometer and temperature was kept at 25 °C during testing. After a three hours rest period ensuring gel setting and structural equilibrium of samples, gels mechanical spectra were recorded using a fixed strain amplitude (values ranging from 10^{-4} to $10^{-2}\%$ depending on the gel elasticity) and ramping the frequency from 100 Hz down to 0.01 Hz. Finally, a sweep in the strain amplitude was performed from 0.001% to 500%, to assess the linear regime of viscoelasticity and the large amplitude oscillatory strain (LAOS) regime. Experiments were repeated with softer gels in order to improve the data from the frequency sweep, using larger strain amplitudes while still belonging to the linear regimes, assessed by previous strain sweeps.

3.9. References

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Chapter 4 - Results and discussion

4.1. Synthesis of the hydrogelators

The hydrogelators methionyldehydrophenylalanine and lysyldehydrophenylalanine *N*-protected with 2-(6-methoxy-2-naphthyl)propionic acid (naproxen, Npx) (figure 4.1) were prepared using a conventional protocol in solution (schemes 4.1 and 4.2).

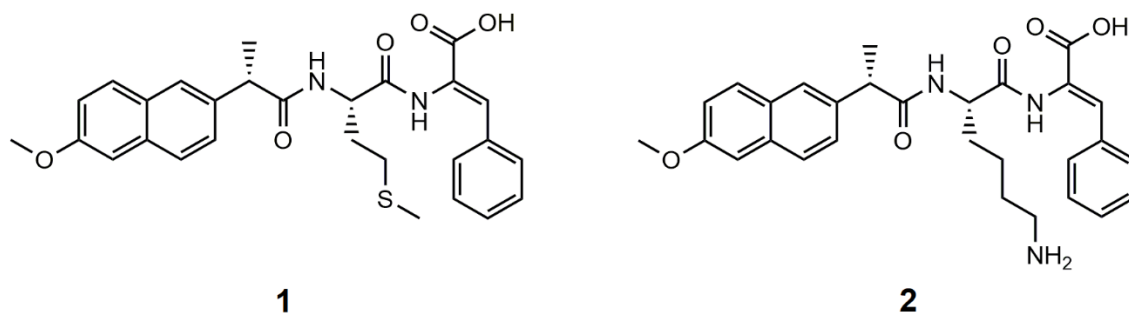
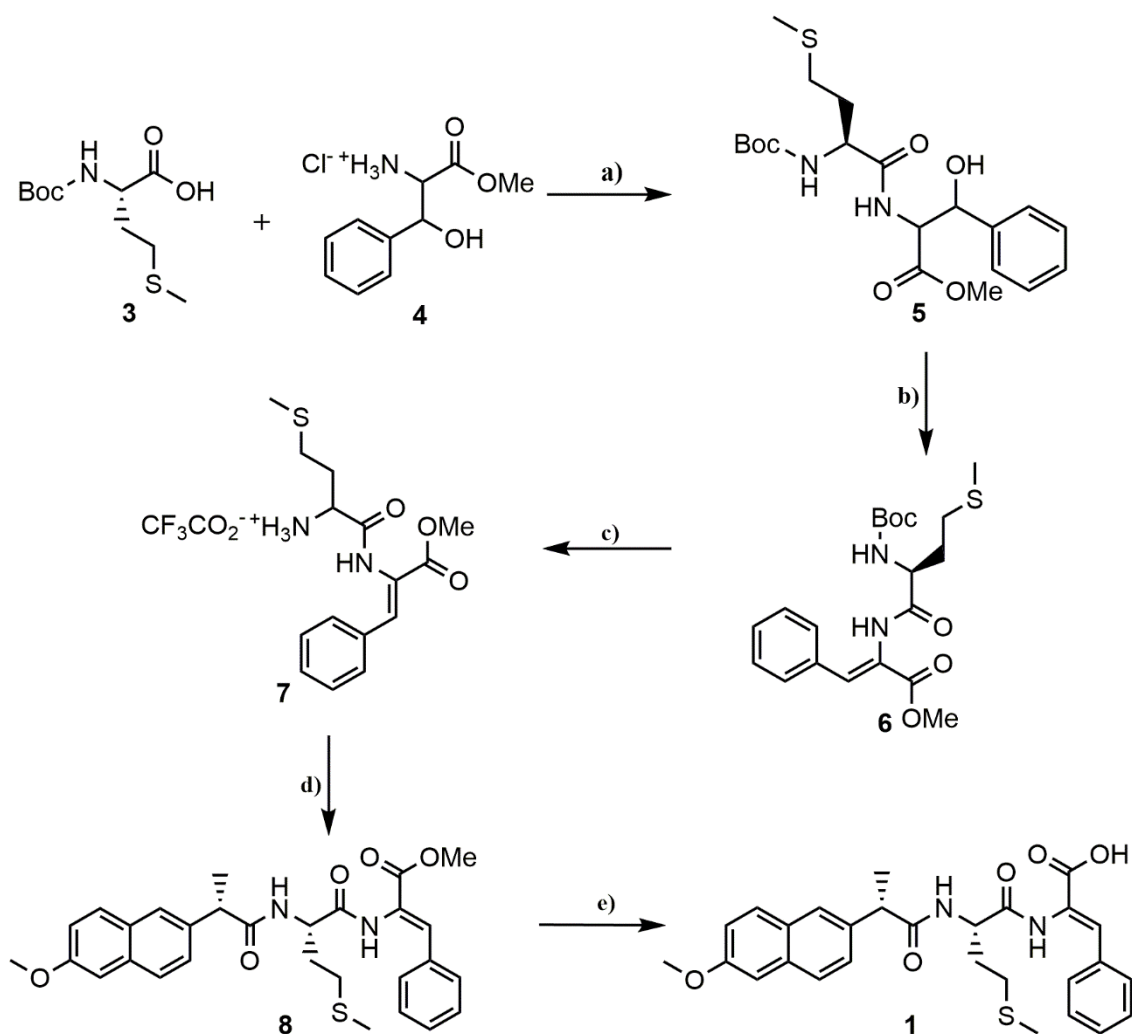


Figure 4.1. Structure of the hydrogelators prepared (Npx-L-Met-Z-ΔPhe-OH and Npx-L-Lys-Z-ΔPhe-OH).

The strategy for the synthesis of the hydrogelator Npx-L-Met-Z-ΔPhe-OH involves the coupling of *N*-*tert*-butoxycarbonyl methionine (Boc-Met-OH, **3**) with the methyl ester of β -hydroxyphenylalanine (H-D,L-Phe(β -OH)-OMe, **4**) (scheme 4.1), using the *N,N*-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBt) procedure. Compound **5** was obtained as diastereomeric mixture in 84% yield. The reaction of the carboxylic acid of compound **3** gives an *O*-acylurea. The latter is very reactive and can lead to the formation of several by-products such as *N*-acylureas or oxazolones [1]. Oxazolones are usually associated with epimerisation [1]. The addition of 1-hydroxybenzotriazole to the reaction mixture reduces epimerization, through the formation of an active ester [1]. Dehydration of the β -hydroxydipeptide, **5** was attained by reaction with di-*tert*-butyl dicarbonate (Boc₂O) in the presence of 4-dimethylaminopyridine (DMAP), followed by treatment with *N,N,N',N'*-tetramethylguanidine (TMG) [2]. Dehydrodipeptide **6** was isolated in a 60% yield. The *N*-*tert*-butoxycarbonyl group of **6** was removed with trifluoroacetic acid (TFA) and *N*-deprotected dehydrodipeptide **7** was coupled with 2-(6-methoxy-2-naphthyl)propionyl chloride. The hydrogelator **1** was obtained after basic cleavage of the methyl ester of compound **8** (scheme 4.1).



Scheme 4.1. Synthesis of hydrogelator **1**. a) DCC, HOBT, Et₃N, ACN, rt; b) (1) Boc₂O, DMAP, dry ACN, rt (2) TFA; c) TFA; d) (S)-(+)-naproxen chloride, Et₃N, DCM, rt; e) (1) NaOH (1 M), 1,4-dioxane, rt, (2) KHSO₄.

In the ¹H NMR spectrum of Npx-L-Met-ZΔPhe-OH, **1** in DMSO (figure 4.2) it is possible to observe a duplet at 1.41 ppm due to the methyl protons of naproxen, two singlets at 2.05 ppm and 3.83 ppm assigned to the SCH₃ and OCH₃ of methionine and naproxen, respectively, and a multiplet between 4.44 ppm and 4.54 ppm due to the α-CH proton of methionine and between 7.08 ppm and 8.26 ppm due to the aromatic protons of naproxen and dehydrophenylalanine. The two signals of the NH protons appear as duplet at 8.27 ppm and as a singlet at 9.52 ppm.

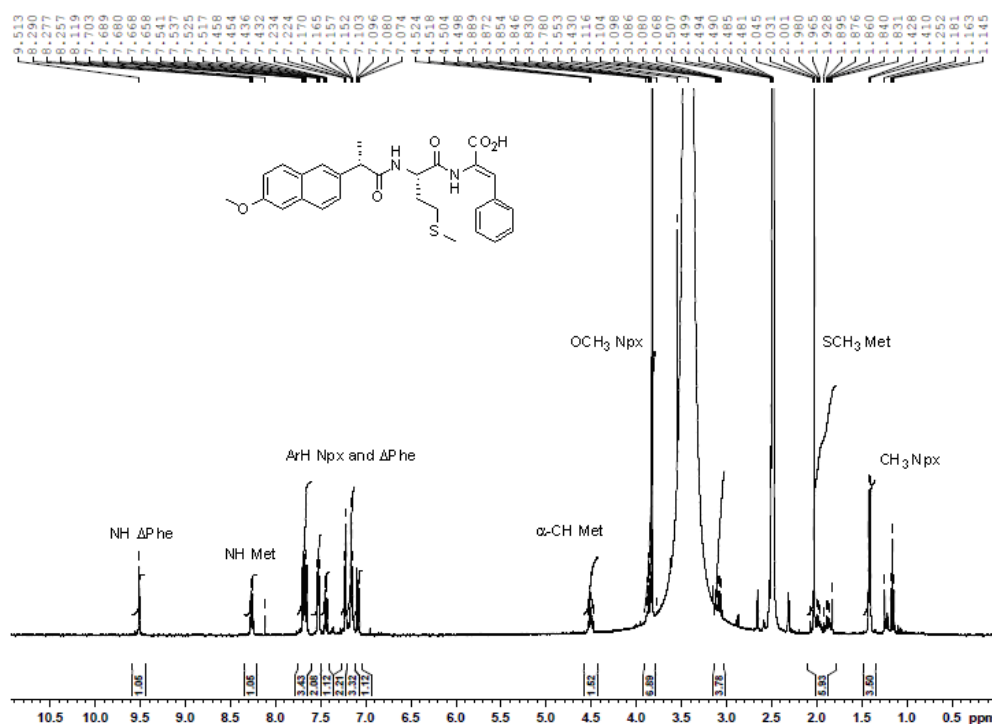
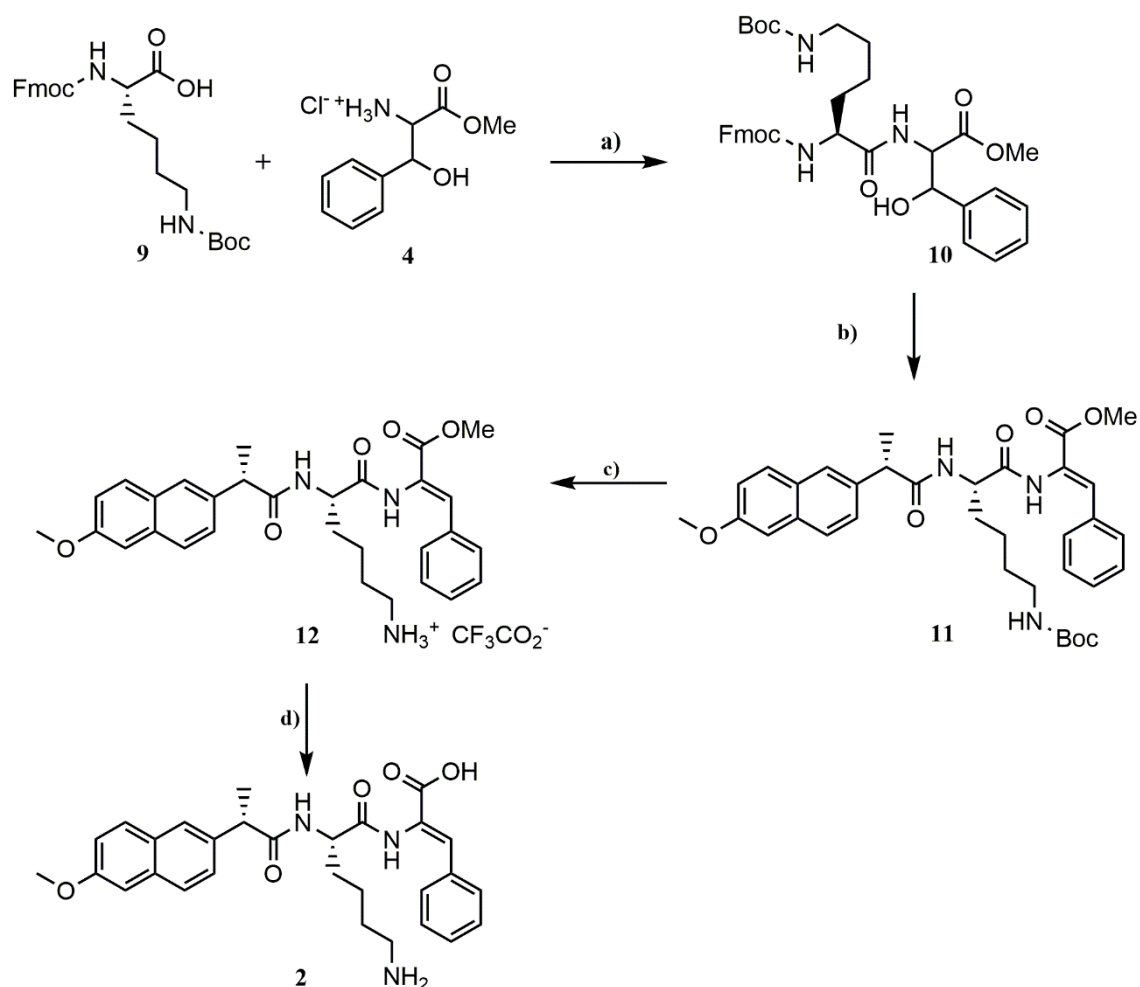


Figure 4.2. ¹H NMR spectrum of Npx-L-Met-ZΔPhe-OH, **1** in DMSO-*d*₆.

The strategy used for the synthesis of the hydrogelator **2**, Npx-L-Lys-ZΔPhe-OH was similar to that described for the preparation of hydrogelator **1** (Npx-L-Met-ZΔPhe-OH) (scheme 4.2). Thus, coupling of fluorenylmethoxycarbonyl-lysine, **9** with the methyl ester of β-hydroxyphenylalanine, **4** was carried using the standard DCC/HOBt procedure. The dipeptide **10** was obtained as a diastereomeric mixture in 93%. *N*-deprotection of **10** followed by reaction with 2-(6-methoxy-2-naphthyl)propionyl chloride gave compound **11**. The latter was dehydrated with Boc₂O/DMAP, followed by TMG. *N*-deprotection **11** followed by basic hydrolysis of the methyl ester afforded hydrogelator **2**.



Scheme 4.2. Synthesis of hydrogelator **2**. a) DCC, HOBT, Et₃N, ACN, rt; b) (1) 2 % piperidine, DMF (2) (S)-(+)-naproxen chloride, Et₃N, DCM, rt (3) Boc₂O, DMAP, dry ACN, rt (4) TMG; c) TFA; e) (1) NaOH (1 M), 1,4-dioxane, rt, (2) KHSO₄.

The ¹H NMR spectrum of the hydrogelator Npx-L-Lys-ZΔPhe-OH, **2** (figure 4.3) shows a singlet at 3.84 ppm due the OCH₃ protons of naproxen, a duplet and a singlet at 8.21 ppm and at 9.34 ppm attributed to α-NH protons of lysine and dehydrophenylalanine, respectively and a broad singlet at 12.63 ppm due to the carboxylic acid proton.

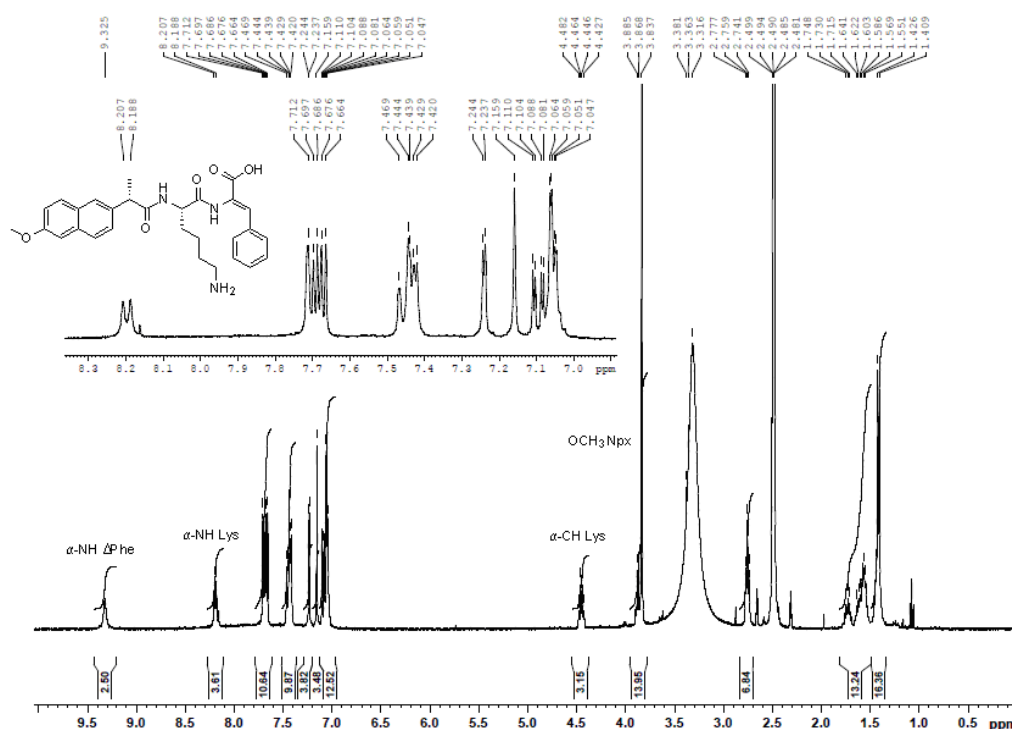


Figure 4.3. ¹H NMR spectrum of Npx-L-Lys-ZΔPhe-OH, 2 in DMSO-*d*₆.

4.2. Hydrogelators characterization

4.2.1. UV-Visible absorption spectra

The hydrogelators UV-visible absorption spectra show the characteristic bands of naphthalene and naphthalene-based compounds (figure 4.4) [3]. The intense band between 210 and 240 nm, with a peak at 233 nm, is associated with the naphthalene long axis π - π^* transition, while the medium band at 260-290 nm (with the absorption maximum at 273 nm) is assigned to the naphthalene short axis π - π^* transition [3,4]. The hydrogelator Npx-L-Lys-ZΔPhe-OH absorption spectrum has a weak band around 333 nm also attributed to naphthalene long axis π - π^* transition [3,4]. Moreover, the phenylalanine absorption, which has a maximum at 260 nm [5], and the peptide backbone n - π^* and π - π^* transition around 210-230 nm and 180-200 nm, respectively, also contribute to the overall spectrum [3]. In both hydrogelators, the absorption spectrum is dominated by naproxen over phenylalanine moiety [5]. However, the backbone-to-naproxen absorption ratio is higher in the methionine containing hydrogelators, thus demonstrating that methionine and lysine introduce different substituent environments and solvent interactions that affect the hydrogelator photophysical properties. Nevertheless, the increased Npx-L-Lys-ZΔPhe-OH absorption around 270 nm can also be contributed by the lysine residue UV-absorption [6].

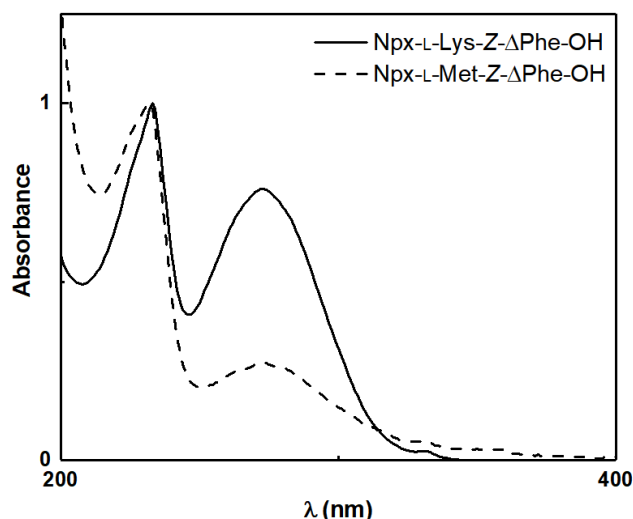


Figure 4.4. Normalized UV-visible absorption spectra of the hydrogelators Npx-L-Met-Z- Δ Phe-OH and Npx-L-Lys-Z- Δ Phe-OH in ethanol (0.2 mM).

4.2.2. Fluorescence assays

4.2.2.1. Self-assembly dependence on pH

Fluorescence spectroscopy was used to determine the pH dependence of the hydrogelators self-assembly by exploring the behaviour of the intrinsic fluorophore naproxen. The hydrogelator solution was excited at 290 nm, as there is no direct excitation of dehydrophenylalanine, besides not changing the emission spectra as the long-axis polarized state is the emitting state [4].

The fluorescence emission spectrum of the hydrogelator Npx-L-Met-Z- Δ Phe-OH shows a main band at 360 nm and a second fluorescence emission band around 450 nm, which are associated to the monomer and aggregate state, respectively (figure 4.5) [3]. The monomer fluorescence emission band shows little pH dependence in the range of pH values from 4 to 7, which exponentially increases at higher pH associated with the increasing deprotonated species, thus favouring the free monomer state. However, it should be noticed that, at pH=4 and 5, a large aggregate to monomer intensity ratio is attained, suggesting that self-assembly into aggregates is favourable at this pH and decreases with increasing pH. Moreover, a predicted pKa of 3.7 was obtained for the terminal carboxylic acid using the MarvinSketch software, which is close the maximum of the ratio I_2/I_1 . Thus, such ratio behaviour suggests that the carboxylic acid pKa must be close to the respective pH, which has been observed for other hydrogelators [3].

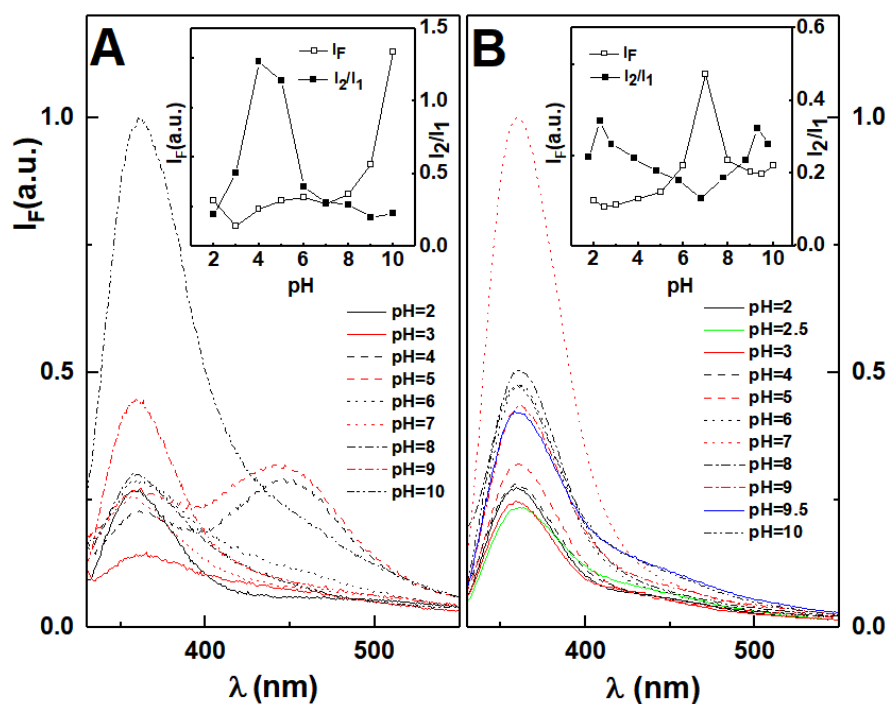


Figure 4.5. Fluorescence emission spectra of (A) Npx-L-Met-ZΔPhe-OH and (B) Npx-L-Lys-ZΔPhe-OH at 2×10^{-6} M in the pH range 2–10 ($\lambda_{exc} = 290$ nm). Insets: Maximum fluorescence intensity (I_F) variation within the pH range and intensity ratio I_2/I_1 of naproxen aggregate (I_2 , 450 nm) and monomer band (I_1 , 360 nm).

The fluorescence emission spectrum of the hydrogelator Npx-L-Lys-ZΔPhe-OH exhibits the monomer band at 360 nm, being followed by a tenuous aggregate fluorescence emission band in the range 430–450 nm. Although the wavelength of the monomer emission band wavelength shows little pH dependence, a strong variation in fluorescence intensity was obtained, which exponentially increases until pH=7 and stabilizes in the pH ranges 2–5 and 8–10. Hereby, the lowest I_2/I_1 ratio is attained at pH=7 and the maximum at pH 2.5 and 9.5. Moreover, the hydrogelator not only establishes ion-dipole interactions, but also hydrogen bonding that might favour the self-assembly. However, a hydrogelator polarity gradient might be required for hydrogelation, considering that a higher aggregate state is attained in the pH ranges 2–3 and 8–10.

4.2.2.2. Critical gelation concentration and hydrogel preparation

The hydrogelator Npx-L-Met-ZΔPhe-OH critical gelation concentration (CGC) assay was carried out in pH=6 buffer, considering that it is closer to the physiological pH, while ensuring the hydrogelator has more aggregates than in pH=7. This is an advantage due to the alteration of aggregation when the hydrogel gets in contact with more acidic environments, such as the tumour region. The increased concentration used in this assay (comparatively to the pH assay) shows no wavelength shifts of the monomer band, while the aggregate band is redshifted to 490 nm. Moreover, in this concentration range,

it is observed an incremental quenching of monomer fluorescence emission associated with a rising aggregate band, where the minimum is attained at 0.7 wt% (figure 4.6). However, aggregate/monomer ratio rapidly increases until 0.5 wt% with no changes on the monomer emission wavelength, suggesting that gelation is attained at this concentration, where the presence of aggregates is higher compared to the lower concentrations. Nevertheless, a stable hydrogel was attained at 0.1 wt%, which was gelated by dilution in a NaOH (1 M) aqueous solution (2 V/V%) followed by pH decrease with glucono- δ -lactone (GdL, 0.4 wt%).

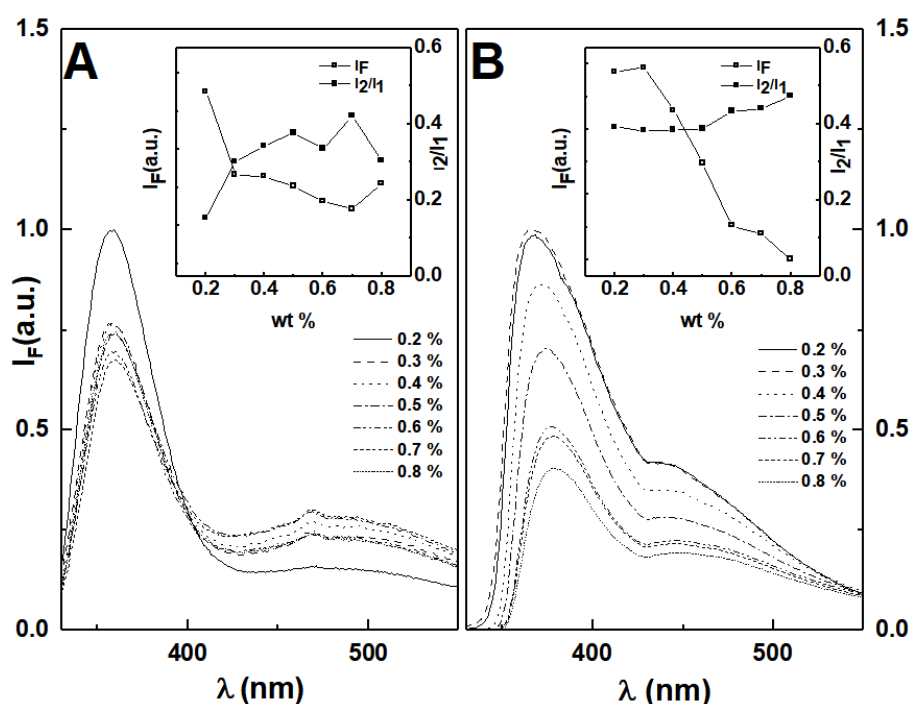


Figure 4.6. Fluorescence emission spectra of (A) Npx-L-Met-Z Δ Phe-OH and (B) Npx-L-Lys-Z Δ Phe-OH in the concentration range 0.2–0.8 wt% at pH=6 and pH=7 (λ_{exc} = 290 nm), respectively. Insets: Maximum fluorescence intensity variation within the pH range and intensity ratio I_2/I_1 of naproxen aggregate (I_2) and monomer band (I_1).

The critical gelation concentration assay for the hydrogelator Npx-L-Lys-Z Δ Phe-OH was carried out according to two different methods. The first method was undertaken in pH=7 buffer, thus guaranteeing a hydrogel close to the physiological pH. Although the aggregate/monomer emission intensity ratio is minimum at this pH, with predominance of the monomer state, the hydrogelator showed little solubility in the required concentration range. Hereby, the hydrogelator was initially dissolved in DMSO (2.5 v/v%), followed by the addition of buffer to a final concentration in the range 0.2–0.8 wt%, having attained a bulk hydrogel state at 0.5 wt%. Hydrogelation was achieved through heating at 60°C after homogenization, followed by sonication for ~10 sec and stabilization. Below the experimentally determined CGC, only aggregate flocks were obtained and a highly viscous solution at 0.4 wt%. In this assay, an increasing concentration is accompanied by a redshift of the fluorescence emission band, associated with a

reduction of the monomer emission band until 0.6 wt% and a larger proportion of the aggregate band afterwards (figure 4.7). Thus, a redshift of the maximum fluorescence emission to 370 nm and of the aggregate band to 450 nm indicates that hydrogelation will occur. At higher hydrogelator concentration (0.8 wt%), the monomer and aggregate bands show no wavelength shifts, forming a highly turbid hydrogel. Nevertheless, it is possible to obtain a homogeneous hydrogel without using the solvent exchange method. Such was attained by dissolving 1 wt% in basic pH, followed by heating at 60 °C and cooling at room temperature, the pH value being 12.7. Therefore, the fluorescence dependence on the hydrogelator concentration was investigated at pH=13 buffer and suggested that gelation might be attained at 0.5 wt%, owing to the higher aggregate/monomer emission intensity ratio as the concentration increases, though it was only attained at 1 wt%. The obtained hydrogels are shown in figure 4.8, where the glass vial flipping confirms the hydrogel formation.

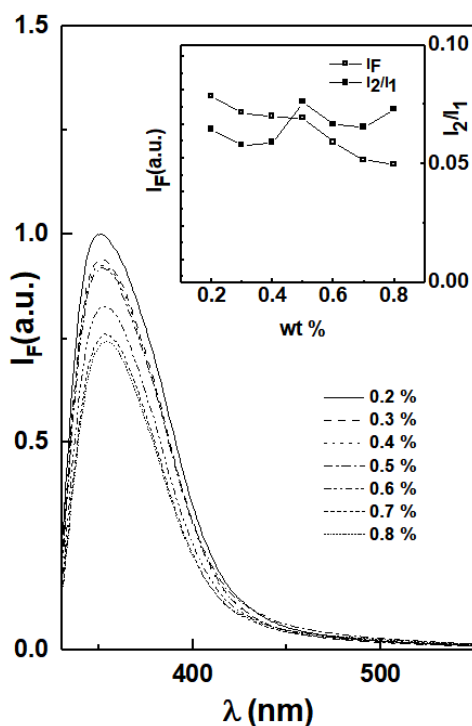


Figure 4.7. Fluorescence emission spectra of the hydrogelator Npx-L-Lys-Z Δ Phe-OH in the concentration range 0.2–0.8 wt% at pH=13 (λ_{exc} = 290 nm). Insets: Maximum fluorescence intensity variation within the pH range and intensity ratio I_2/I_1 of naproxen aggregate (I_2) and monomer band (I_1).

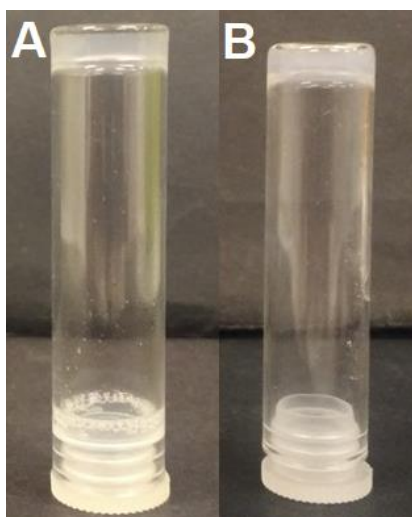


Figure 4.8. Hydrogel formation confirmation for the (A) Npx-L-Lys-ZΔPhe-OH (1 wt%, pH=13 buffer) and (B) Npx-L-Met-ZΔPhe-OH (1 wt%, 2 v/v% 1 M NaOH, 0.4 wt% GdL).

It was noticed that the lysine derivative-based hydrogel shows a highly viscous behaviour similar to a semisolid hydrogel, while the methionine derivative and the lysine derivative-based hydrogel obtained through the solvent-exchange method behave as solid-like hydrogels. Hereby, the possibility of preparing the hydrogel at the desired pH by the solvent-exchange method allows adapting it to the circumstances, besides the pH responsive property. However, the use of organic solvent inherently leads to a loss of biocompatibility, but the same argument could be used for the heating-cooling method that required high pH for proper solubilization. Considering this disadvantage, a method was developed to neutralize the hydrogel, thus increasing its inherent biocompatibility. For that, an equal volume of aqueous solution of HCl 0.2 M is added to the hydrogel, left standing for 1 min, replaced with pH=7 buffer that is left for 20 min, and then followed by 4 cycles of buffer replacement. The confirmation of hydrogel neutralization is shown in figure 4.9. However, the hydrogel loses its thermal reversibility.

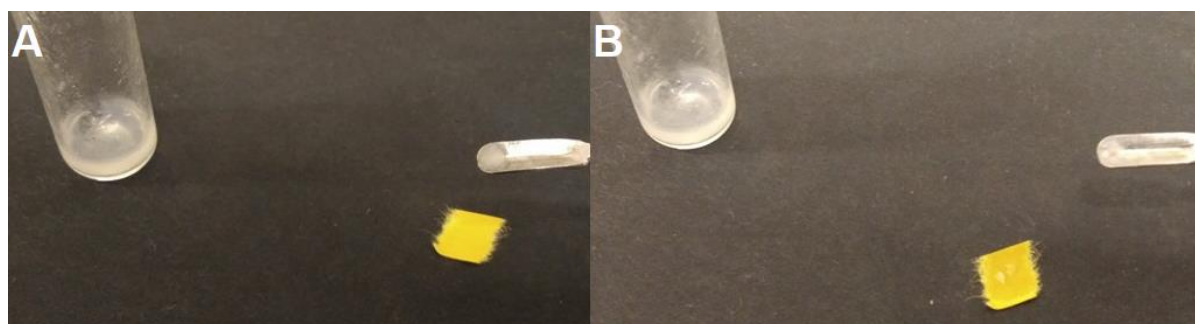


Figure 4.9. Demonstration of the hydrogel neutralization confirmation (from A to B).

4.2.2.3. Critical aggregation concentration

The critical aggregation concentration of both hydrogelators was also assessed by fluorescence spectroscopy. As the concentration increases, an enhancement of monomer fluorescence emission band is observed until a maximum, which decreases afterwards. Hereby, the maximum fluorescence emission is associated with the concentration at which self-assembly/aggregation takes place at room temperature, without applying any stimulus. A decrease of monomer fluorescence occurs for higher concentration until the monomer state is favoured again, owing to the high energy cost required to attain the self-assembled hierarchical state (hydrogel). For the hydrogelator Npx-L-Met-Z Δ Phe-OH, a maximum was obtained at 0.025 nM, while the hydrogelator Npx-L-Lys-Z Δ Phe-OH shows it at 0.028 nM at pH=13 and 0.32 nM at pH=7 (figure 4.10). The latter higher concentration value might be associated with the fact that hydrogelator Npx-L-Lys-Z Δ Phe-OH shows its higher solubility at pH=7, thus requiring a larger concentration to favour the formation of aggregates. However, such high solubility might have not been observed on the CGC assays, due to the zwitterionic state requiring a precise ionic strength. Moreover, a predicted log P of 4.12 and 3.34 were determined for Npx-L-Met-Z Δ Phe-OH and Npx-L-Lys-Z Δ Phe-OH, respectively, further demonstrating that the former has a higher propensity to aggregate that correlates with the obtained results for the critical aggregation concentration assay. The lysine derivative might have its aggregation between 0.028 nM and 0.11 nM, while methionine has its aggregation close to 0.025 nM owing to the higher hydrophobicity. Such results also demonstrate that log P is a good predictor for the design of hydrogelators.

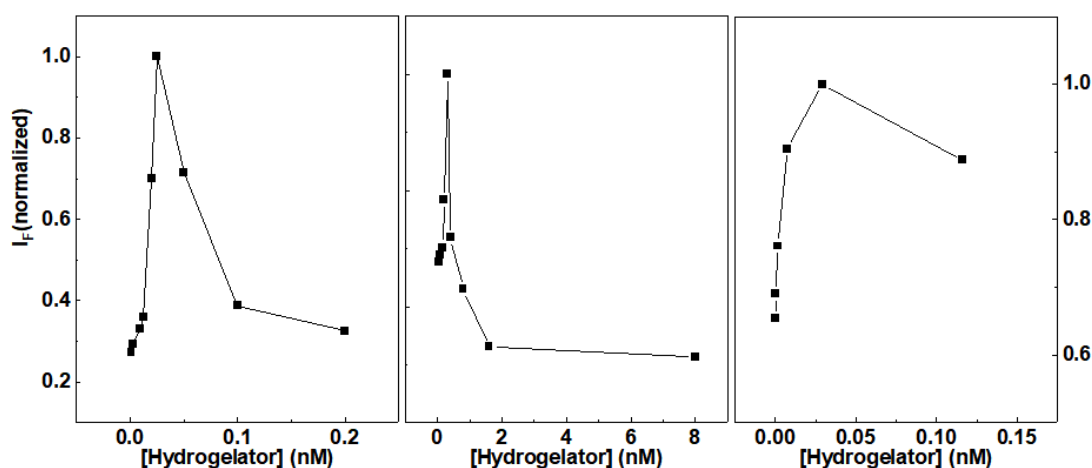


Figure 4.10. Fluorescence emission assay for determination of critical aggregation concentration of (left) Npx-L-Met-Z Δ Phe-OH at pH=6, (middle) Npx-L-Lys-Z Δ Phe-OH at pH=7 and (right) pH=13 (λ_{exc} = 290 nm), respectively.

4.2.3. Computational studies

4.2.3.1. Molecular properties

To get an insight into the hydrogelators chemical reactivity, the occurrence of intramolecular charge transfer and the nature of the electronic transitions, molecular quantum chemical calculations were carried out using naproxen as a benchmark. A dipole moment of 1.39 D was obtained, which agrees with previously reported values for naproxen geometry optimization using other basis sets (table 4.1) [7]. Moreover, the calculated excitation wavelengths for the first three excited states are in close agreement with the experimental wavelengths for naproxen in water and ethanol reported in reference [4], though the transition S_0 - S_3 shows the largest shift from the reported values.

Table 4.1. Excited states experimental and calculated wavelength (nm), electronic transition contributions and dipole moment of naproxen (Debye). Abbreviations: exp: experimental; calc: calculated; trans: main transitions; λ : wavelength.

Excited State	Exp* λ (nm)	Calc λ (nm)	Trans	Dipole Moment (Debye)				
				6-311G+(d,p)		6-31G(d) [†]		6-31G(d,p) [†]
				B3LYP	HF	B3LYP	HF	B3LYP
S ₁	312	319	H→L					
S ₂	285	285.72	H→L+1	1.39	1.23	1.30	1.23	1.30
S ₃	221	257.29	H→L+2					

[†]Dipole moments obtained from reference [7]. *Experimental wavelengths obtained from reference [4].

The electronic transition from the ground to the first excited state is mainly described by one electron excitation from HOMO to LUMO, where the associated energy and energy gap is responsible for the chemical and spectroscopic properties [8]. A HOMO and LUMO energy of -5.73 eV and -1.37 eV were obtained, respectively. Thus, the associated energy gap of 4.36 eV suggests that an intramolecular charge transfer interaction occurs.

The molecular orbitals of naproxen associated with the electronic transitions are represented in figure 4.11. The HOMO charge density is mainly distributed along the naphthalene, methoxy and carbonyl moieties, where transition to the LUMO delocalizes the density to the naphthalene ring, showing nodes between α - and β -carbon atoms. In the LUMO+1, a large charge density is observed in the α -carbon atom, while in the LUMO+2 the orbital wavefunctions have their orbitals mainly in the naphthalene moiety. All the excitations involve mainly $\pi \rightarrow \pi^*$ transitions from HOMO to LUMO.

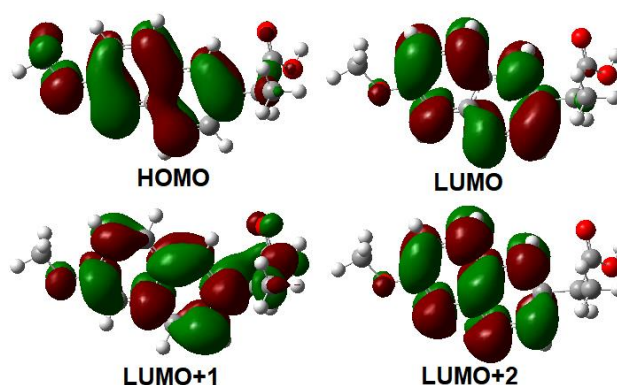


Figure 4.11. Main molecular orbitals associated with electronic transitions of naproxen. Green and red regions represent the positive and negative lobes of the calculated molecular orbitals.

The molecular electrostatic potential (MEP) consists on a plot of net electrostatic effect at a point in space produced by the total charge distribution of a molecule, providing a mean to visually understand the relative molecular polarity, size, shape, charge density, and site of chemical reactivity [8-10]. The potential increases in the order red < orange < yellow < green < blue, where the blue represents regions of proton repulsion and red corresponds to regions of protons attraction by the atomic nuclei. The highest and lowest electrophilicity is observed for the OH group and O atom of the carboxylic moiety region, respectively (figure 4.12).

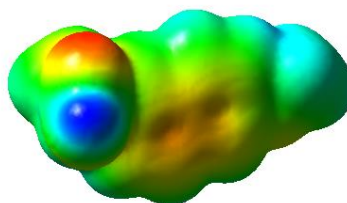


Figure 4.12. Representation of the electrostatic potential surface of naproxen.

The dipole moment obtained for hydrogelators reveals its higher polarity (comparatively to naproxen) that was introduced by the peptide sequence. The Npx-L-Lys-Z Δ Phe-OH attains the highest dipole moment (table 4.2), which can be associated to a higher separation and uneven distribution of charge along the hydrogelator. This high dipole moment in the neutral hydrogelator state suggests that a strong dipole interaction might occur between the hydrogelator molecules that will strongly favour its electrostatic aggregation. Indeed, such was observed experimentally in the critical aggregation concentration assay, as a stabilization was required prior to fluorescence measurement of the Npx-L-Lys-Z Δ Phe-OH due to instantly forming aggregates upon addition to a buffer solution. On the other hand, the dipole moment might also be used as a predictor for the hydrogels hierarchical self-assembly, as the lower dipole moment correlates with a lower critical aggregation and gelation concentration of Npx-L-Met-Z Δ Phe-OH, while the

opposite was obtained for Npx-L-Lys-ZΔPhe-OH, *i.e.* the latter requires a higher concentration to attain the hydrogel state.

The calculated wavelengths are in general redshifted comparatively to the experimentally obtained, which could be associated with specific solvent-solute interactions apart from the accuracy of the used methods, considering that a lower level of theory basis set was used for the peptide backbone both in the optimization and energy calculation.

Table 4.2. Excited states experimental and calculated wavelength (nm), the main electronic transition contributions and dipole moment of the hydrogelators (Debye). Abbreviations: exp: experimental; calc: calculated; trans: main transitions; λ: wavelength.

Excited State	Exp λ (nm)	Calc λ (nm)	Trans	Dipole Moment (Debye)	Hydrogelator
				6-311G+(d,p)/3-21G	
				B3LYP/HF	
S ₁	331	310.75	H-2→L+1	2.26	Npx-L-Met-ZΔPhe-OH
S ₂	273	303.97	H→L		
S ₃	232	297.61	H→L+1		
S ₁	331	323.25	H→L	4.95	Npx-L-Lys-ZΔPhe-OH
S ₂	273	303.08	H-2→L		
S ₃	233	300.26	H→L+1		

An insight on the main molecular orbitals contributing to the transition from the ground state to the excited states is provided by the representation in figures 4.13 and 4.14, for the hydrogelators Npx-L-Met-ZΔPhe-OH and Npx-L-Lys-ZΔPhe-OH, respectively.

The transition to the first excited state (310.75 nm) of hydrogelator Npx-L-Met-ZΔPhe-OH is mainly described by the transition from HOMO-2 to LUMO+1, which occurs through the delocalization of charge density from the dehydrophenylalanine aromatic ring to the naproxen moiety. The second excited state, with absorption wavelength at 303.97 nm, is mainly contributed by the HOMO to LUMO transition, where the charge density is dislocated from the naproxen moiety to the dehydrophenylalanine residue and the peptide backbone, whereas in the transition to the third excited state (297.61 nm) a charge density remains accumulated in the naproxen moiety (figure 4.13). Thus, the obtained electronic transitions suggest the occurrence of intramolecular charge transfer, which is also verified for the other hydrogelator.

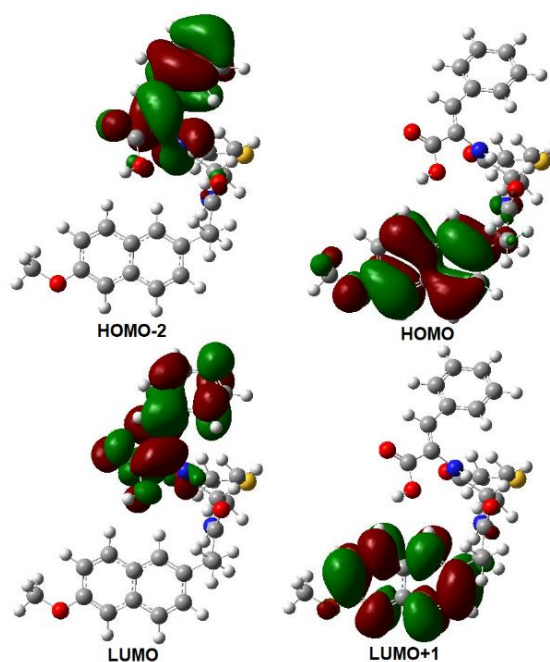


Figure 4.13. Main molecular orbitals associated with electronic transitions of Npx-L-Met-Z Δ Phe-OH. Green and red regions represent the positive and negative lobes of the calculated molecular orbitals.

In the hydrogelator Npx-L-Lys-Z Δ Phe-OH, the excitation to the first excited state (323.25 nm) presents, as main contribution, the transition from HOMO to LUMO, where the charge density dislocates from the naproxen moiety and lysine amine to the peptide backbone and dehydrophenylalanine residue. The excitation to the second excited state (303.08 nm) is mainly contributed by the HOMO-2 to LUMO transition, where the HOMO-2 is characterized by the charge density distributed along the naproxen moiety and dehydrophenylalanine residue, which transition to the LUMO occurs through accumulation of the charge density on the peptide backbone and dehydrophenylalanine residue. In the transition to the third excited state (300.26 nm), the charge density is observed to remain distributed along the naproxen moiety (figure 4.14).

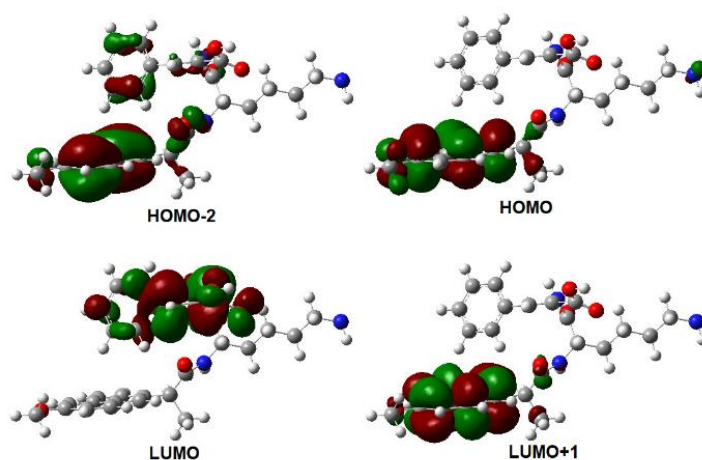


Figure 4.14. Main molecular orbitals associated with electronic transitions of Npx-L-Lys-Z Δ Phe-OH. Green and red regions represent the positive and negative lobes of the calculated molecular orbitals.

The peptide sequence conjugated to naproxen did change the electronic behaviour of the naproxen moiety, where the electron density delocalization from naproxen to the dehydrophenylalanine residue in the HOMO to LUMO transition seems to be a common behaviour in the first three electronic transitions, apart from the transitions where the charge density is delocalized from dehydrophenylalanine to the naproxen moiety or remains distributed along the latter. Hereby, considering that the frontier molecular orbitals dictate many of the molecular properties and that the HOMO-LUMO has a similar behaviour in the two hydrogelators, the molecular transition suggests that they will have similar molecular properties, where the peptide sequence and the dehydroamino acid might introduce some changes in the hydrogelators chemical and electronic behaviour, although the coupled fluorophore (naproxen) has the greatest impact over it owing to its role in all the electronic transitions.

The intramolecular charge transfer character observed in the molecular orbitals mainly contributing for the electronic transitions is also evidenced by the large HOMO-LUMO gap obtained for each hydrogelator, which is 4.51 eV and 4.27 eV for Npx-L-Met-ZΔPhe-OH and Npx-L-Lys-ZΔPhe-OH, respectively, suggesting that conjugating a dipeptide to naproxen does not dramatically change the molecular electronic properties.

Thus, to evaluate the peptides effect on the global chemical reactivity of the hydrogelators, descriptors of the hard and soft, acid and bases (HSAB) theory were used, which describes the hardness and softness in terms of the ionization potential and electron affinity that can be related to the frontier molecular orbitals using the Koopman's theorem for closed-shell molecules [7,11,12]. The classification according to soft or hard is related to the displacement or delocalization of the electron density to form new covalent bonds, i.e. the polarizability, where soft stands for easily polarizable and hard for less distorted charge densities [11]. Hereby, a small HOMO-LUMO gap corresponds to a soft molecule, while a large means a hard molecule, which can be related to the molecule stability, as a smaller gap results in higher reactivity [12]. The hardness (η) and softness (σ) can be determined according to the equations:

$$\eta = \frac{(E_{LUMO} - E_{HOMO})}{2} \quad (4.1)$$

$$\sigma = \frac{1}{\eta} \quad (4.2)$$

where E_{HOMO} and E_{LUMO} are the HOMO and LUMO molecular orbital energies, respectively.

Moreover, it is possible to combine softness and chemical potential in a single parameter, the electrophilic index (ω) that is defined as:

$$\omega = \frac{\mu^2}{2\eta} \quad (4.3)$$

where μ represents the chemical potential, i.e. the easiness a molecule can undergo chemical change, which can be described by the equation:

$$\mu = \frac{(E_{LUMO} + E_{HOMO})}{2} \quad (4.4)$$

The obtained results for the molecular properties are represented in table 4.3.

Table 4.3. Naproxen and hydrogelators chemical potential (μ), hardness (η), softness (σ) and electrophilic index (ω) descriptors.

Compound	μ (eV)	η (eV)	σ (eV ⁻¹)	ω (eV)
Npx	-3.55	2.18	0.46	2.89
Npx-L-Met-Z Δ Phe-OH	-3.36	2.26	0.44	2.50
Npx-L-Lys-Z Δ Phe-OH	-3.31	2.14	0.47	2.58

Both the hydrogelators show a lesser negative chemical potential than naproxen, with the methionine-based hydrogelator showing the lowest. The negative value suggests that compounds are stable and will not decompose spontaneously into the constitutive elements. Moreover, the hydrogelator Npx-L-Lys-Z Δ Phe-OH shows the lowest hardness, thus being the most reactive compound, while Npx-L-Met-Z Δ Phe-OH is the most stable and with the lowest electrophilic power. Hereby, the HSAB descriptors for the studied hydrogelators suggest that the dehydrodipeptide can change the chemical properties by reducing the resistance to changes of electron number and distribution, as indicated by the global hardness, which was expected due to the smaller HOMO to LUMO gap. Nevertheless, the calculated parameters show that the conjugated peptide sequences did not introduce any major instability to the naproxen moiety, with a chemical stability comparable to naproxen, and that it is mainly responsible by the electronic properties, as previously predicted by the analysis of molecular orbitals involved in the electronic transitions.

To further understand the influence of the peptide epitope on the hydrogelator molecular reactivity, the molecular electrostatic potentials (MEP) were obtained, being represented in figure 4.15. The Npx-L-Met-Z Δ Phe-OH side chain shows that methionine sulphur and aliphatic carbons have little electronegativity difference and are low prone to reaction. In all hydrogelators, the more nucleophilic regions are associated to the carbonyl oxygen, apart from the amine nitrogen atom in the hydrogelator Npx-L-Lys-Z Δ Phe-OH due to the free electron pairs, while a higher electrophilicity is associated to the amide NH groups.

The electrostatic potential obtained for both hydrogelators can offer some insight on the interaction between the cyclooxygenase recognition cavity and the naproxen moiety, as the MEP is a representation

of how the molecule vicinity is perceived. Considering that some changes occurred in the naproxen electrostatic potential surface, such suggests that it may have some effect on the protein-ligand interaction compared to the unconjugated naproxen. Moreover, the dehydrophenylalanine aromatic ring orientation of the hydrogelator Npx-L-Lys-Z Δ Phe-OH might hinder the entrance and interaction with the active site, i.e. can hamper the naproxen moiety from effectively reaching the active site cavity by requiring higher torsional energy. Nevertheless, the interaction with the active site cavity will be evaluated in the following section.

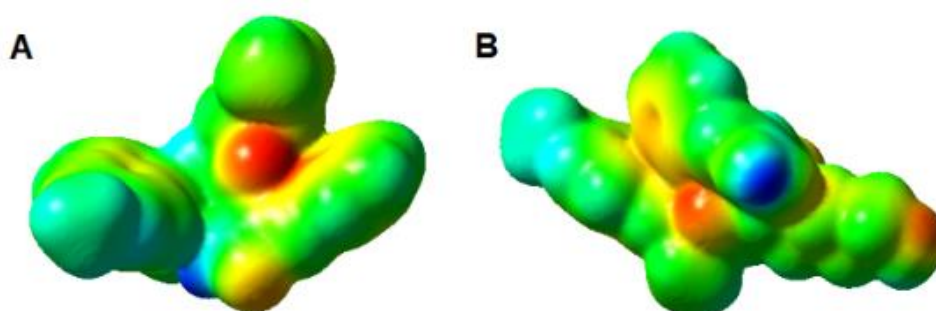


Figure 4.15. Electrostatic potential surface of (A) Npx-L-Met-Z Δ Phe-OH and (B) Npx-L-Lys-Z Δ Phe-OH.

4.2.3.2. Docking studies in cyclooxygenase-1 and -2

Direct docking of the crystallographic naproxen was performed to evaluate the reliability of the defined protocol, where the best scoring pose (lowest binding energy) was superimposed over the original PDB structure. The root mean square deviation was calculated to measure the docking prediction accuracy, having obtained an overall error of 0.61 Å. Polar interactions between naproxen carboxylate moiety and Tyr³⁵⁵ hydroxyl group and Arg¹²⁰ guanidinium moiety, which are responsible for naproxen stabilization in the binding pocket through hydrogen bonding [13], as well as the respective measured distances, are represented in figure 4.16. Moreover, the hydrophobic residues Leu³⁵², Val³⁴⁹, Ala⁵²⁷, and Val⁵²³ are also represented in the active site, due to their role in the stabilization of the ligand through hydrophobic interactions [13].

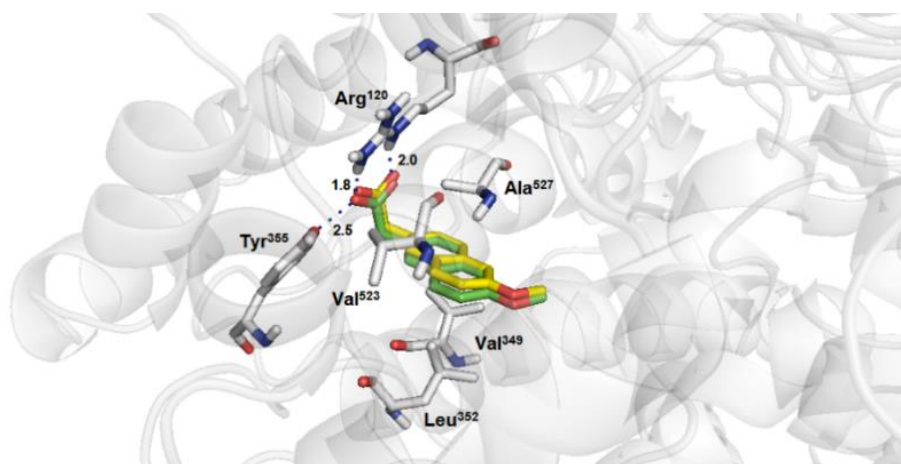


Figure 4.16. Representation of the crystallographic (green) and direct docking (yellow) naproxen conformation in the active site of cyclooxygenase-2 and polar interactions distances (Å) obtained from PyMOL software.

Cross docking between hydrogelators and COX-2 (cyclooxygenase-2) followed the same procedure applied to naproxen direct docking, to evaluate the binding affinity for the COX-2 active site and which parameters influence the hydrogelators stabilization in the binding pocket. The obtained lowest free energies of binding, intermolecular energy contributions (van der Waals, hydrogen bonding, desolvation and electrostatic energy) and the predicted inhibitory constants are represented in table 4.4.

Table 4.4. Naproxen (Npx) and hydrogelators estimated inhibition constant, estimated free energy of binding, intermolecular energy and its contributions (van der Waals, hydrogen bonding, desolvation energy and electrostatic energy) on the interaction with cyclooxygenase-2 obtained from AutoDockTools-1.5.6 software.

Compounds	Npx	Npx-L-Met-ZΔPhe-OH	Npx-L-Lys-ZΔPhe-OH
Estimated inhibition constant (μM)	0.89	3.57	3.90
Estimated free energy of binding (kcal/mol)	-8.25	-7.43	-7.38
Intermolecular energy (kcal/mol)	-9.15	-11.01	-11.55
Van der Waals, hydrogen bonding and desolvation energy (kcal/mol)	-8.09	-10.93	-11.08
Electrostatic energy (kcal/mol)	-1.06	-0.08	-0.48

All the hydrogelators show a lower affinity towards COX-2 than naproxen, as it is evidenced by the lower free energy of binding and higher inhibition constant, i.e. the dissociation constant. Moreover, the lysine-based hydrogelator has a more unfavourable interaction with the active site, though the difference is not significant, suggesting that the possible hindering by lysine residue might be counter-balanced by electrostatic and favourable intermolecular interactions. The highest contribution is associated to the

intermolecular energy, which partitioning in the respective components reveals that van der Waals, hydrogen bonds and desolvation are the main interactions leading towards the complex formation, while the electrostatic energy has a lesser relevant contribution. Hereby, the former contribution suggests that the affinity might be related to a higher number of hydrogen bonds established between the polar groups of the hydrogelators dipeptide backbone and the polar amino acids of the binding pocket. Another possibility is the favourable stabilization of the hydrogelator along the cavity leading to the binding pocket through van der Waals and aromatic interactions. Therefore, to properly evaluate the parameters that influence the hydrogelators affinity towards the binding pocket, the visual representation of the best docking poses and the amino acids involved in the stabilization in the binding pocket were assessed through PyMOL software (figure 4.17). The methionine and lysine side chains affected the orientation of naproxen moiety in the binding cavity. Moreover, the results suggest that the van der Waals interactions have a prominent role, as the hydrogelator Npx-L-Met-Z Δ Phe-OH shows less hydrogen bonds than the other hydrogelator, but still has a similar affinity towards the binding cavity, which could be associated to the proper stabilization by hydrophobic residues. The higher number of hydrogen bonds in Npx-L-Lys-Z Δ Phe-OH contributes to a more favourable intermolecular interaction, where the penalty is associated with the steric hindrance by the lysine residue and the inadequate orientation and stabilization of the naproxen residue.

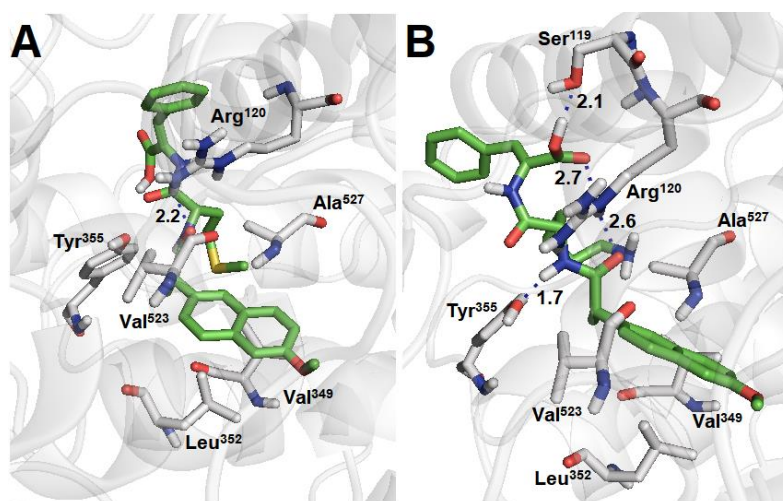


Figure 4.17. Representation of the best docking poses and polar interactions distances (Å) for the hydrogelators (A) Npx-L-Met-Z Δ Phe-OH and (B) Npx-L-Lys-Z Δ Phe-OH in COX-2 binding pocket obtained from PyMOL software.

Furthermore, to evaluate the effect of the peptide backbone on the specificity of naproxen towards COX-2 and COX-1, docking was also performed for the latter. The direct docking of the crystallographic flurbiprofen attained a root-mean-square deviation (RMSD) of 0.765 Å thus making the defined protocol suitable. Apart from the hydrogen bonding established between flurbiprofen carboxylate moiety and Tyr³⁵⁵

hydroxyl group and Arg¹²⁰ guanidinium moiety (figure 4.18), the hydrophobic residues Leu³⁵², Val³⁴⁹, Ala⁵²⁷, Trp³⁸⁷, Leu⁵³¹, and Ser⁵³⁰ are also represented in the active site owing to their role in the ligand stabilization by hydrophobic interactions.

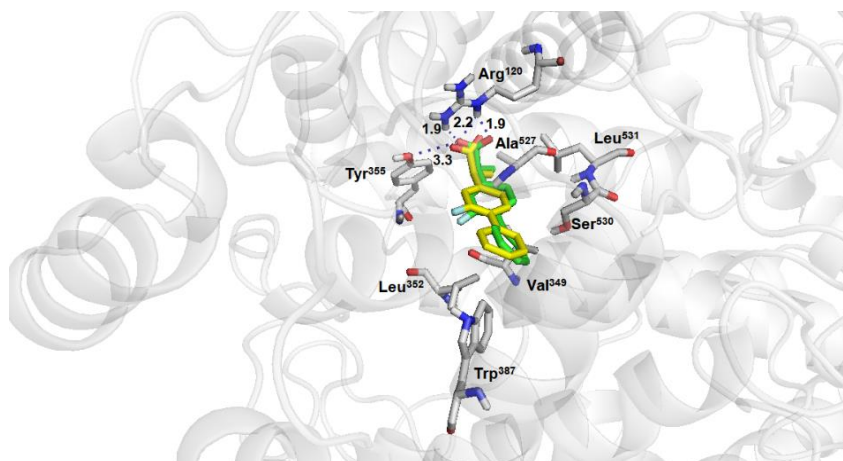


Figure 4.18. Representation of the crystallographic (green) and direct docking (yellow) flurbiprofen conformation in the active site of cyclooxygenase-1 and polar interactions distances (Å) obtained from PyMOL software.

The intermolecular energy contributions (table 4.5) reveal that van der Waals, hydrogen bonding and desolvation are the leading interactions to ligand-receptor complex, as previously observed for COX-2. Moreover, the electrostatic interaction greatly favours the Npx-L-Lys-ZΔPhe-OH binding, compared to the obtained value for the COX-2 binding, which results on a higher affinity (lower inhibition constant) than the other hydrogelator. Hereby, the results suggest that the peptide backbone did change the naproxen specificity towards the active site. The hydrogelator Npx-L-Lys-ZΔPhe-OH is expected to have a greater affinity towards COX-1, while Npx-L-Met-ZΔPhe-OH has a higher specificity for COX-2. Therefore, such affinity differences introduced by the peptide backbone could provide a means to modulate the specificity towards the desired COX enzyme.

Table 4.5. Flurbiprofen (Flp) and hydrogelators estimated inhibition constant, estimated free energy of binding, intermolecular energy and its contributions (van der Waals, hydrogen bonding, desolvation energy and electrostatic energy) on the interaction with cyclooxygenase-1 obtained from AutoDockTools-1.5.6 software.

Compounds	Flp	Npx-L-Met-ZΔPhe-OH	Npx-L-Lys-ZΔPhe-OH
Estimated inhibition constant (μM)	0.213	16.69	3.11
Estimated free energy of binding (kcal/mol)	-9.10	-6.52	-7.51
Intermolecular energy (kcal/mol)	-10.00	-10.10	-11.69
Van der Waals, hydrogen bonding and desolvation energy (kcal/mol)	9.24	-9.79	-10.22
Electrostatic energy (kcal/mol)	-0.76	-0.31	-2.45

The methionine and lysine side chains do not show the same steric hindrance effect previously described for COX-2 binding cavity, where a notorious change of the naproxen orientation was highlighted (figure 4.19). Furthermore, hydrogen bonding interactions are established between the lysine residue of the hydrogelator Npx-L-Lys-ZΔPhe-OH and the Pro⁸⁹ carbonyl oxygen atom and Glu⁵²⁴ carboxyl moiety, which contribute for a better stabilization that is not obtainable in Npx-L-Met-ZΔPhe-OH due to the methionine hydrophobic side chain. Hereby, hydrophilic amino acids, such as lysine, rather than bulk hydrophobic side chain, might contribute to a better stabilization of the dehydrodipeptide-based hydrogelators in COX-1 binding pocket.

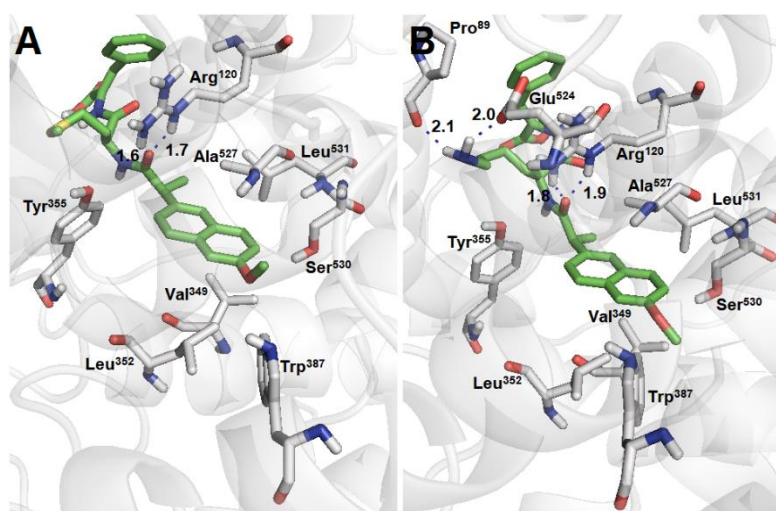


Figure 4.19. Representation of the best docking poses and polar interactions distances ($\text{\AA}$) for the hydrogelators (A) Npx-L-Met-ZΔPhe-OH and (B) Npx-L-Lys-ZΔPhe-OH in COX-1 binding pocket obtained from PyMOL software.

4.3. Synthesis of magnetic/plasmonic nanoparticles

4.3.1. Core/shell manganese ferrite/gold nanoparticles

The method used for the synthesis of manganese ferrite/gold core/shell nanoparticles explores the similarity between the crystal lattices of MnFe_2O_4 and Au nanoparticles, i.e. when two materials have similar crystal lattices, one can be uniformly deposited on the other through epitaxial growth (figure 2.20) [14]. Hereby, gold was deposited on the coprecipitated MnFe_2O_4 nanoparticles through reduction of chloroauric acid (HAuCl_4) by glycerol at high temperature (200 °C). The purification required magnetic separation, as the method utilized is prone to the production of self-nucleated gold nanoparticles.

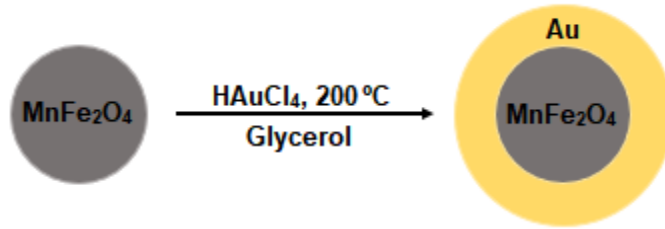


Figure 4.20. Schematic representation of the manganese ferrite/gold core/shell nanoparticles. Adapted from [15].

The gold shell resulted in the appearance of a wide plasmon band with maximum absorption at 556 nm (figure 4.21), which could be associated to a shell larger than 5 nm, being in accordance with the commonly reported surface plasmon resonance frequencies for core/shell nanoparticles (below 600 nm) [14,16]. From the Tauc plot, the optical band gap (E_g) of the manganese ferrite nanoparticles can be estimated according to the equation [17]:

$$(\alpha h\nu)^{\frac{1}{n}} \propto (h\nu - E_g) \quad (4.5)$$

where α is the absorption coefficient, h is the Plank constant, ν is the light frequency and n is a coefficient that depends whether the transition nature is direct ($n=1/2$) or indirect ($n=2$). A linear relation was obtained for an indirect transition, as previously reported by Rodrigues *et al.* [18]. A gap energy of 1.09 eV was obtained, which is similar to previous reported values of 1.08 [18] and 0.98 eV [19].

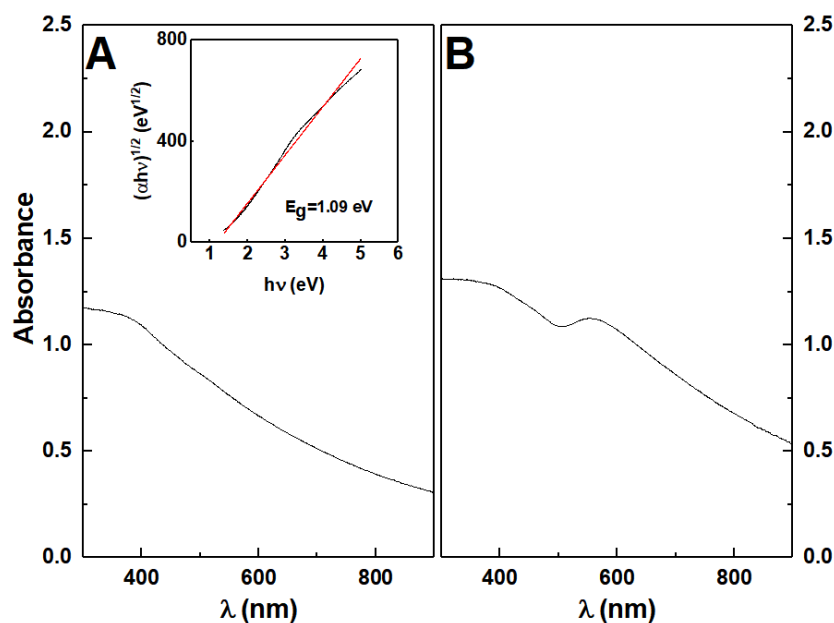


Figure 4.21. Absorption spectra of (A) MnFe_2O_4 and (B) manganese ferrite/gold core/shell nanoparticles. Inset: Tauc plot of the respective nanoparticles.

Considering the bare surface of core/shell nanoparticles, the titration with both hydrogelators (figure 4.22) induced a reduction of global absorbance (absorbance at 600 nm), higher gold to manganese ferrite absorbance ratio, and a redshift and widening of the gold plasmon band, thus suggesting that the hydrogelators promoted some aggregation of the core/shell nanoparticles.

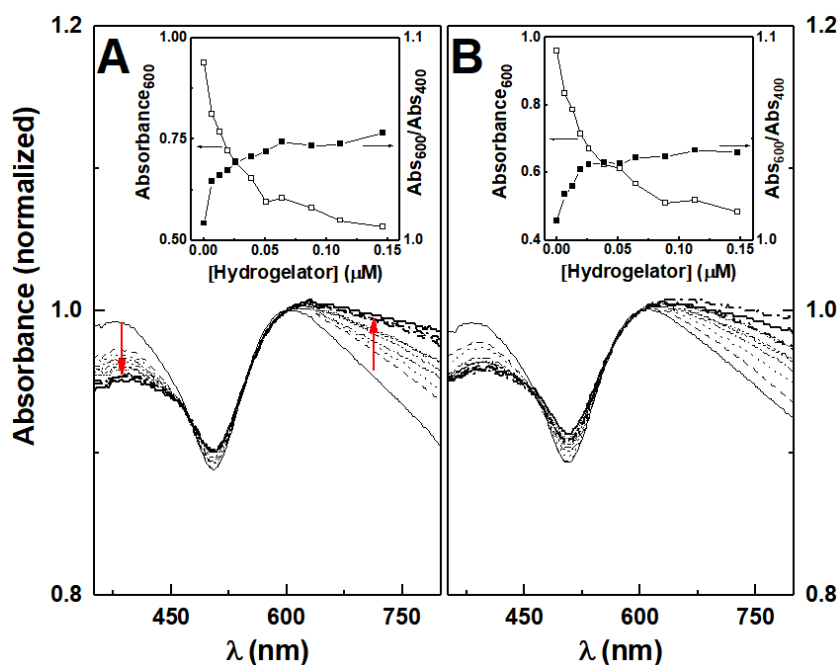


Figure 4.22. Normalized (at 600 nm) absorption spectra of (A) manganese ferrite/gold core/shell nanoparticles (0.018 wt%) titrated with the hydrogelator (A) Npx-L-Met-Z Δ Phe-OH and (B) Npx-L-Lys-Z Δ Phe-OH. Inset: Absorbance at 600 nm and ratio of absorbance at 600 nm to 400 nm.

4.3.2. Gold-decorated manganese ferrite nanoparticles

The method used in the synthesis of gold nanoparticles required a thiolate, 3-mercaptopropionic acid (MPA) that acted as a stabilizer, owing to the strong affinity of sulphur towards sulphur-gold coordination bonds and the reducing agent, sodium borohydride. The reduction of Au (III) with NaBH_4 generally gives nanoparticles with sizes smaller than 10 nm and the stabilization by a thiolate (MPA) ensures the gold nanoparticle dispersion for weeks. The method was adapted from [20] where the mass quantities were kept constant but for 50 ml, sodium borohydride was dissolved in NaOH 1M (as it was hard to solubilize in ethanol), and the nanoparticles were close to 5 nm if left reacting for 30 min. Upon addition of the sodium borohydride solution, the reaction occurred instantaneously and a black/brown precipitate was formed. The synthesis of gold-decorated manganese ferrite nanoparticles followed the method reported in [20]. Hereby, MnFe_2O_4 are initially reacted with 1,1'-carbonyldiimidazole (CDI) in DMSO, where a nucleophilic attack to CDI carbonyl carbon occurs, which results into a carbamate linkage and imidazole as a secondary product. After removing the secondary products and unreacted CDI, the activated nanoparticles are reacted with the MPA-functionalized gold nanoparticles (figure 4.23), resulting into gold nanoparticles bond to manganese ferrite nanoparticles through an ester bond. However, the MPA-gold decorated manganese ferrite nanoparticles showed some sedimentation during gelation, thus MPA was further coupled to 2-aminoethanol (MEA) through CDI coupling and washed with water. The amount of gold nanoparticles was estimated based on the required number of gold nanoparticles to cover the surface area of manganese ferrite nanoparticles, where an estimated size of 5 nm was used for gold nanoparticles, and a size of 25 nm was used for manganese ferrite nanoparticles, as it has been the usual average size obtained for this method, as reported in reference [18]. Moreover, a density of 19.4 g/mL and 5.04 g/mL were used for gold and manganese ferrite nanoparticles, respectively [21].

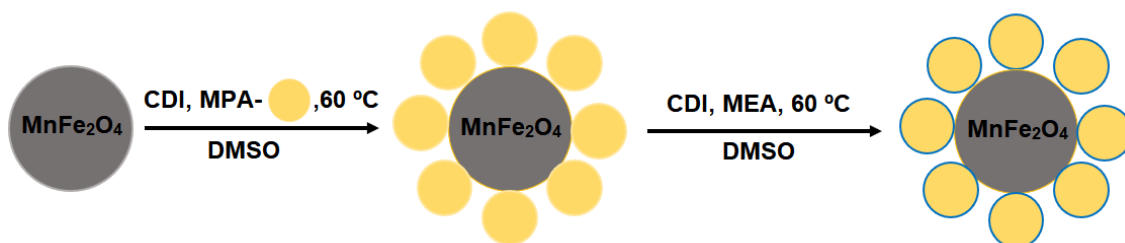


Figure 4.23. Schematic representation of MPA (3-mercaptopropionic acid)-functionalized gold nanoparticles and MEA (2-aminoethanol) coupling to manganese ferrite nanoparticles through 1,1'-carbonyldiimidazole method.

The absorption spectra (figure 4.24) show the SPR band characteristic of gold nanoparticles centred around 530 nm, which can be associated with a size around 5-10 nm, being similar to gold nanoparticles capped with thiol ligands published in other works [20,22]. Moreover, the precipitate colour is not associated with aggregation of nanoparticles, as no absorption band around 700 nm is observed [20]. Comparatively to manganese ferrite nanoparticles, the contribution from the gold nanoparticle plasmon is observed around 550 nm, thus suggesting that gold nanoparticles have been effectively bonded to manganese ferrite nanoparticles, while the absorbance close to 800 nm might be associated to aggregates. Nevertheless, the obtained spectra demonstrate that gold nanoparticles are indeed small, owing to the manganese ferrite nanoparticles spectral predominance over the gold nanoparticles.

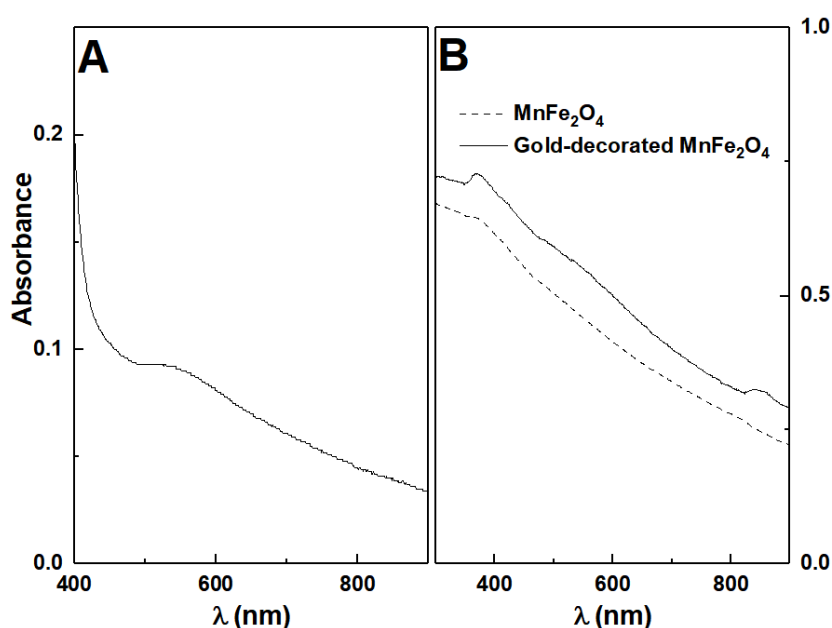


Figure 4.24. Absorption spectra of (A) MPA-functionalized gold nanoparticles and (B) gold-decorated manganese ferrite nanoparticles compared to manganese ferrite nanoparticles dispersed in ultrapure water.

Considering that the carboxylic moiety of 3-mercaptopropionic acid can coordinate Fe(III) ions on the nanoparticles surface, an alternative method was evaluated for the synthesis of gold-decorated nanoparticles. Hereby, manganese ferrite nanoparticles were added to a solution of MPA-gold nanoparticles in an aprotic solvent (DMSO, DMF and 1,4-dioxane) in a proportion of 1:1 at above 80 °C to favour dehydration of manganese ferrite nanoparticles surface. The reaction was undertaken on a water bath without sonication, as the presence of the latter did not allow the formation of the labile coordination bond. The UV-Vis absorption spectra of the obtained nanoparticles are shown in figure 4.25. From the synthesis in DMSO, two types of decorated nanoparticles were obtained, one that displays a fast-magnetic response, with a well-defined gold plasmon band, and a second that was obtained through precipitation of the nanoparticles by freezing the solution, showing a large gold plasmon band, thus suggesting the

existence of large clusters of aggregated gold nanoparticles. The nanoparticles synthesised in DMF and dioxane showed a large absorption band that did not allow a clear distinction between gold and manganese ferrite nanoparticles absorption owing to aggregation. Thus, it was required the addition of NaOH to increase the electron density on gold nanoparticles and improve its dispersion in water. Moreover, the gold-decorated nanoparticles can be destroyed upon application of ultrasonication for one hour and loss of gold nanoparticles over time was observed when incubated in pH=12 buffer, as a consequence of the labile carboxylic acid-Fe(III) coordination bond, that resulted in a spectrum similar to the manganese ferrite nanoparticles. Nevertheless, such lability is interesting for photothermia, as its breakage triggered by the gold nanoparticles heating is expected to further enhance the plasmonic heating, owing to the nanoparticles not having the plasmon hampered by manganese ferrite nanoparticles.

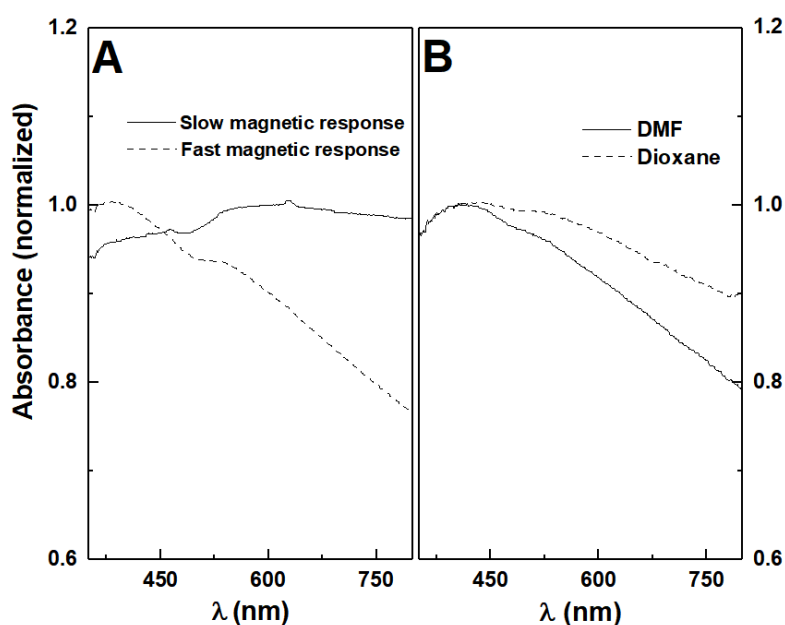


Figure 4.25. Normalized absorption spectra of water dispersed gold-decorated manganese ferrite nanoparticles synthesised in (A) DMSO, (B) DMF and dioxane. The absorption spectra of DMF and dioxane gold-decorated manganese ferrite nanoparticles were obtained after addition of NaOH 1 M.

4.3.3. X-ray diffraction characterization

The X-ray diffraction (XRD) pattern of manganese ferrite nanoparticles presents well-defined peaks (figure 4.26), suggesting a structure of crystalline nature, even without calcination and after addition of the gold shell, as previously reported [18,21]. The gold-decorated manganese ferrite nanoparticles show a neat amorphous contribution associated with the organic layer of the MPA-capped gold nanoparticles. Nevertheless, diffraction peaks of the gold nanoparticles are observed at $2\theta = 38.3$ and 44.5 , while the ones for the gold shell are centred at $2\theta = 38.1$ and 44.4 , corresponding to the (1 1 1) and (2 0 0) plans of the crystalline structure, respectively. Distinctive diffraction peaks of the MnFe_2O_4 crystalline structure are observed at $2\theta = 29.7$ (2 2 0), 34.9 (3 1 1), 36.5 (2 2 2), 42.5 (4 0 0), 52.7 (4 2 2), and 56.2 (3 3 3) (5 1 1).

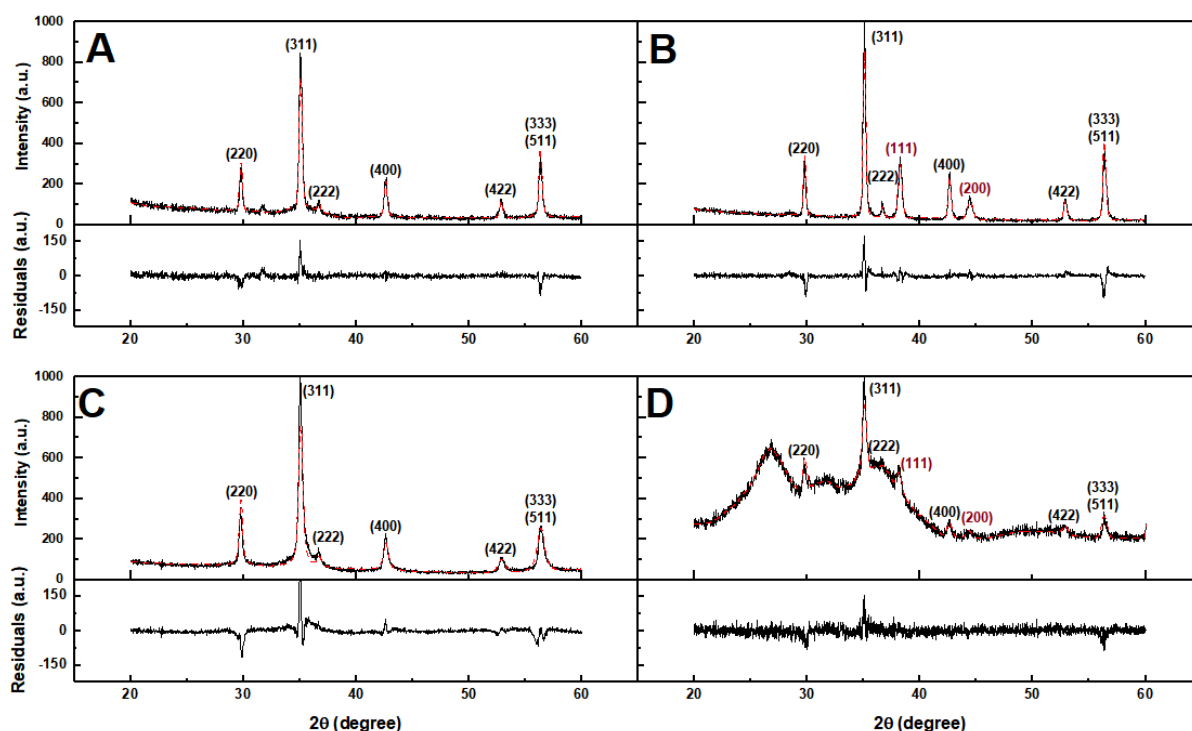


Figure 4.26. Nanoparticles X-ray diffraction pattern (A) before and (B) after addition of a gold shell to the manganese ferrite nanoparticles and (C) before and (D) after decoration of the manganese ferrite nanoparticles with gold nanoparticles. The black line represents the experimental pattern and the dotted red line is the fitted pattern.

Rietveld analysis was carried out using a background defined by linear interpolation between a set of points at constant scattering angles and refinable intensities. The calculated parameters and phase sizes are represented in table 4.6. The manganese ferrite phases have an average size of approximately 13 nm, while the gold phase in the core/shell nanoparticles has been estimated for 19.6 nm. Considering that the determined size of the gold shell approximates a sphere diameter, the volume was recalculated for a shell. The estimated shell size is of 4.2 nm and the overall nanoparticle size is 21.4 nm. Rietveld

analysis of the gold-decorated nanoparticles is highly affected by the organic coating contribution, as evidenced by the large R_f value. The percentages of each phase determined by matching the respective contribution in the software *Match!* suggest that gold nanoparticles are just a small fraction of the whole nanoparticles, as opposed to the core/shell nanoparticles, which will induce a lesser impact on the magnetic properties of the manganese ferrite core than the latter.

Table 4.6. X-ray diffraction Rietveld refinement calculated parameters R_f and χ^2 , phase sizes and percentages. Legend: CS: used in core/shell nanoparticles; D: used in gold-decorated nanoparticles; $\text{MnFe}_2\text{O}_4/\text{Au}$: core/shell nanoparticles; $\text{MnFe}_2\text{O}_4@\text{Au}$: gold-decorated MnFe_2O_4 nanoparticles.

Nanoparticles	Intensity percentages		Phase size		Parameters	
	MnFe_2O_4	Au	MnFe_2O_4	Au	R_f	χ^2
MnFe_2O_4 (CS)	100	-	13.2	-	9.8	1.1
$\text{MnFe}_2\text{O}_4/\text{Au}$	85.5	14.5	13.3	19.6	9.0	1.1
MnFe_2O_4 (D)	100	-	13.1	-	9.8	1.4
$\text{MnFe}_2\text{O}_4@\text{Au}$	94	6	12.7	15.0	14.0	1.3

4.4. Development of magnetogels

The magnetogels were prepared at 1 wt% of hydrogelator to keep a common concentration of nanoparticles at 10 m/m%. The hydrogelator Npx-L-Met-Z Δ Phe-OH was able to attain hydrogelation even in the presence of nanoparticles, though it required a higher concentration of GdL (0.5 wt%) compared to the hydrogel without nanoparticles (0.4 wt%). Moreover, the addition of nanoparticles prior to pH rising of the aqueous hydrogelator solution resulted in the formation of irreversible aggregates, while a stable solution was attained when the nanoparticles were added after dissolution of the hydrogelator in basic pH. The hydrogelator Npx-L-Lys-Z Δ Phe-OH also hydrogelated at 1 wt% in the presence of 10 m/m% of nanoparticles through a heating-cooling cycle.

The hydrogelators naproxen moiety intrinsic fluorescence allows following the incorporation of the magnetic nanoparticles into the hydrogels through changes on the photophysical behaviour. All emission fluorescence spectra show a maximum emission at 360 nm, associated with the monomer state of the naproxen moiety, while the aggregates emission band around 450 nm (described in reference [3]) is only observed on Npx-L-Met-Z Δ Phe-OH hydrogel/magnetogel (figure 4.27). This difference on the presence of aggregate emission bands could be associated with the conditions and methods utilized for triggering hydrogelation, or the methionine/lysine side chains affecting the naproxen moieties proximity, i.e. it is expected that the Npx-L-Met-Z Δ Phe-OH hydrogelators might be more closed packed in the fibres than the Npx-L-Lys-Z Δ Phe-OH ones.

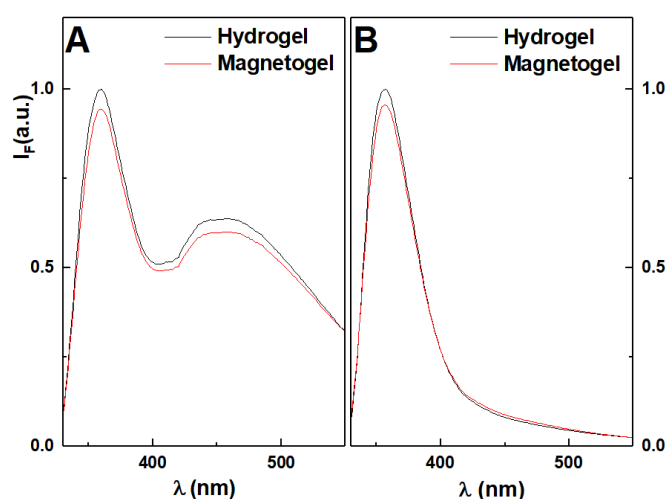


Figure 4.27. Hydrogel and magnetogel (core/shell, 10 m/m%) normalized fluorescence emission spectra of the hydrogelators (A) Npx-L-Met-Z Δ Phe-OH (1 wt%, 2 v/v% 1 M NaOH, 0.5 wt% GdL) and (B) Npx-L-Lys-Z Δ Phe-OH (1 wt%, pH=13 buffer).

Upon the introduction of magnetic nanoparticles, various photophysical effects come into play, mainly the fluorescence quenching. Such effect can be a result from the electronic energy transfer to the

nanoparticles owing to its wide absorption spectrum, but also due to the heavy atom effect, where the presence of heavy atoms enhances the spin-orbit interaction that favours the intersystem crossing [23]. Hereby, an increased energy loss through non-radiative processes induces a decrease of fluorescence emission. However, more effects are included due to the localized surface plasmon of the gold shell. The near-field enhancement close to the nanoparticles will increase absorption and fluorescence, where the latter is enhanced owing to the coupling of the lowest order plasmonic modes (electric dipolar mode) to the far-field [24-26]. Plasmon modes can also inhibit fluorescence at close distances due to coupling to higher order plasmonic modes that are optically dark, which combined with the charge transfer when the fluorophore is close to the vicinity of the nanoparticle, make fluorescence strongly quenched [25]. Furthermore, all the effects are further maximized upon resonance of the fluorophores transition frequencies with the plasmon modes. Nevertheless, considering that even the aggregate fluorescence of the hydrogelator Npx-L-Met-Z Δ Phe-OH is not enough to account for a proper excitation of the plasmonic nanoparticles modes, the obtained fluorescence quenching evidences the predomination of the non-radiative mechanisms on the magnetogels. On the other hand, the introduction of gold-decorated manganese ferrite nanoparticles did not produce the same fluorescence quenching observed for the one containing core/shell nanoparticles, which can be accounted for the surface functional groups hindering the proximity of the fibres (figure 4.28). Yet, the results suggest a modification on the structure of the fibres, as denoted by the higher monomer to aggregate intensity ratio and the blueshift in monomer emission. The magnetogel based on the hydrogelator Npx-L-Lys-Z Δ Phe-OH shows the same quenching effect previously observed for the magnetogels containing core/shell nanoparticles, without significantly changing the fluorescence emission and band shape.

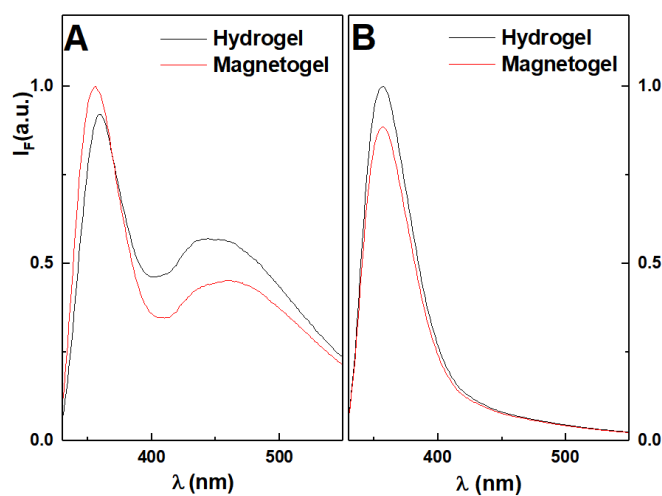


Figure 4.28. Hydrogel and magnetogel (gold-decorated manganese ferrite, 10 m/m%) normalized fluorescence emission spectra of the hydrogelators (A) Npx-L-Met-Z Δ Phe-OH (1 wt%, 2 v/v% 1 M NaOH, 0.5 wt% GdL) and (B) Npx-L-Lys-Z Δ Phe-OH (1 wt%, pH=13 buffer).

Interestingly, if the naproxen moieties integrating the fibrils are equally spaced and the fibrils are assumed to be continuous, then fluorescence anisotropy values (table 4.7) suggest that the hydrogel fibrils of Npx-L-Met-Z Δ Phe-OH have suffered a structural change with the introduction of the core/shell nanoparticles. Such is also verified for the Npx-L-Lys-Z Δ Phe-OH hydrogel despite its lower anisotropy, which is an indicator that the presence of colloidal entities does affect the fibrils three-dimensional organization.

Table 4.7 Naproxen fluorescence anisotropy values in the hydrogel and magnetogel.

Compound	Fluorescence anisotropy, <i>r</i>		
	Hydrogel	Core/shell	Decorated
Npx-L-Met-Z Δ Phe-OH	0.113	0.043	0.035
Npx-L-Lys-Z Δ Phe-OH	0.031	0.014	0.010

4.4.1. Evaluation of hydrogels and magnetogels microstructure

4.4.1.1. FTIR-ATR spectroscopy

The microstructure of hydrogels and magnetogels was investigated by FTIR-ATR technique. The amide I region comprises the 1700-1600 cm⁻¹ region, where the main contribution for infrared absorption is the C=O stretching, which is sensible to the protein secondary structure owing to the hydrogen bonding pattern and molecular geometry (figure 4.29). However, this variety of frequencies leads to a featureless band consisting of various component bands that are instrumentally unresolved, thus requiring the use of mathematical methods, such as second-derivative or Fourier self-deconvolution to identify overlapping bands and assign the corresponding bands [27-30]. From the two methods, the second-derivative is particularly interesting, as it does not require any kind of arbitrary half-bandwidths and enhancement factors [31].

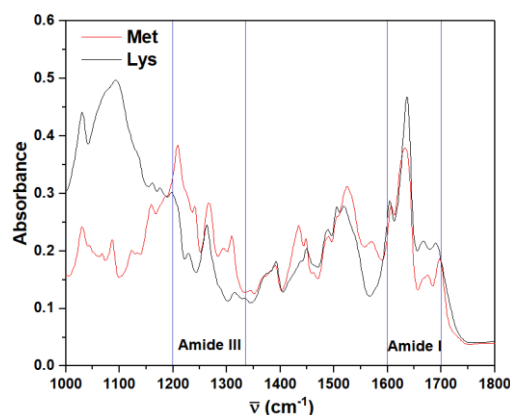


Figure 4.29. Infrared absorption spectra of the hydrogelator Npx-L-Met-ZΔPhe-OH and Npx-L-Lys-ZΔPhe-OH solids and representation of the amide I and III regions.

Hereby, considering that the gels amide I region shows a featureless band (figure 4.30), different approaches were used to quantitatively determine each secondary structure contribution for the absorption band. A first approach was the direct curve fitting to the amide I after baseline correction; the second method was curve fitting to the second derivative peaks; and the third one was the curve fitting to amide III region (1220-1330 cm^{-1}) that is mainly contributed by the in-phase combination of N-H in-plane bending and C-N stretching vibration. The percentage of secondary structure of the amide I band is affected by the water absorption overlapping, which also worsens the ambiguity in the assignment of random coil (1642-1657 cm^{-1}) and α -helix (1648-1657 cm^{-1}) bands [28]. The amide III region does not suffer from the water absorption signal and has a better localization of the different amide bond vibrations contributions. However, amide III lacks its accuracy due to the overlapping of other vibrational groups and the weak distinction between random coil (1270-1255 cm^{-1}) and turn (1295-1270 cm^{-1}) frequencies.

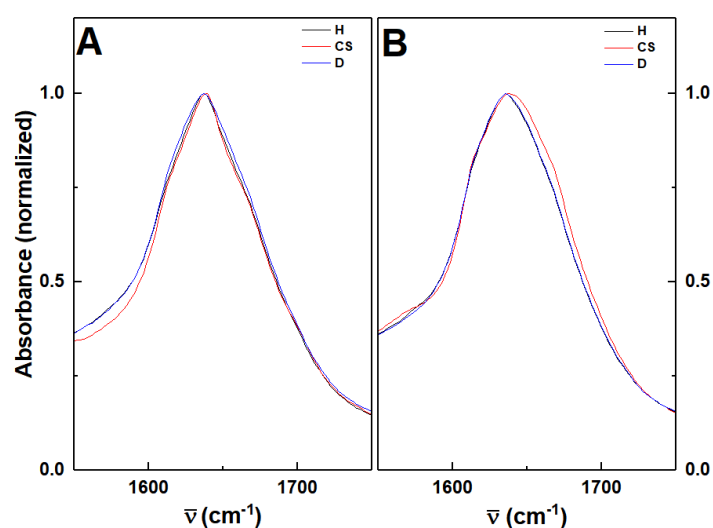


Figure 4.30. Hydrogel (H), core/shell (10 m/m%) (CS) and gold-decorated manganese ferrite nanoparticles (10 m/m%) (D) based magnetogels amide I infrared absorption spectra of the hydrogelator (A) Npx-L-Met-ZΔPhe-OH (1 wt%, 2 v/v% 1 M NaOH, 0.5 wt% GdL) and (B) Npx-L-Lys-ZΔPhe-OH (1 wt%, pH=13).

The second derivative showed a peak around 1625 cm⁻¹ and 1639-1641 cm⁻¹ that can be assigned to β -sheet and random coil, respectively (see figure A1 and A2 in annex). Other peaks were identified at 1668 cm⁻¹ and 1685 cm⁻¹, being associated with the presence of turns and loops, while a peak at 1699 cm⁻¹ might be a result of strong hydrogen bonds between β -sheets or β -strands aggregates [28]. The major difference is that magnetogels have a decrease in intensity of the ordered structure bands, suggesting that, in the presence of nanoparticles, the hydrogelators are more randomly oriented. However, the analysis of amide I band is affected by the presence of the water bending vibration, which overlaps the secondary structure vibrations. Also, the water interaction leads to a broadening of the signals, thus complicating the interpretation, as it might lead to secondary structure percentages with higher error and ambiguity in the assignment of overlapping random coil and α -helix bands. Hereby, the quantitative analysis was carried out considering that the α -helix does not have a significant contribution when dealing with small structures, and that only β -sheet, random coil and turns are present. Alternatively, the amide III region was explored, as it is not contaminated by the water signal and the different amide bond vibrations contributions are better localized. However, the amide III region might be interfered by other vibrational groups, apart from the weak distinction between random coil and turn frequencies, and the low signal intensity. Tables 4.8 and 4.9 display the percentages of secondary structures obtained for each hydrogel and magnetogel.

Table 4.8. Percentage of secondary structures in hydrogels and magnetogels according to the general range reported in [28-32], obtained through curve-fitting of the amide I band (AI), amide I second derivative (ASD) and amide III band (AIII) of the hydrogelator Npx-L-Met-Z Δ Phe-OH. Legend: OC: ordered structures (α -helix and β -sheet); UC: unordered structures (turn and random coil).

System	H			CS			D		
Method	AI	ASD	AIII	AI	ASD	AIII	AI	ASD	AIII
α -helix	0	0	9.8	0	0	13.3	0	0	12.8
β -sheet	45.8	28.1	19.7	41.6	24.3	13.0	34.4	25.2	13.0
Turn	19.9	17.0	23.0	9.4	24.5	33.9	41.2	16.3	32.0
Random coil	34.3	54.9	47.4	49.0	44.2	39.8	24.4	58.5	42.2
OC	45.8	28.1	29.6	41.6	26.1	26.3	34.4	25.2	25.8
UC	54.2	71.9	70.4	58.4	73.9	73.7	65.6	74.8	74.2
χ^2	4 $\times$ 10 ⁻⁷	1 $\times$ 10 ⁻¹¹	3 $\times$ 10 ⁻⁸	4 $\times$ 10 ⁻⁸	3 $\times$ 10 ⁻¹¹	7 $\times$ 10 ⁻¹⁰	2 $\times$ 10 ⁻⁷	6 $\times$ 10 ⁻¹²	1 $\times$ 10 ⁻¹⁰

The results obtained for both hydrogelators show a close agreement between the amide III and second derivative curve fitting method, mainly when the percentages of ordered (α -helix and β -sheet) and unordered (random coil and turns) structures are compared. Overall, a reduction of ordered secondary structures was obtained on the magnetogels when compared to the hydrogels, which is accompanied by

an increase of unordered structures. Hereby, a higher percentage of unordered structures might reduce the compactness of the well-ordered self-assembled structures, owing to the lack of ordered hydrogen bonding, which not only might induce thicker fibres than in the hydrogels, but also a less stable matrix.

Although amide I (comparatively to amide III) shows a higher percentage of β -sheets, which can be associated with water contamination, it is not possible to conclude the effect of decorated nanoparticles on the magnetogel based on Npx-L-Lys-Z Δ Phe-OH through the amide I region, as the hydrogel does not show large differences in secondary structure percentages from the respective magnetogel. Moreover, the results also suggest that the second amino acid has a small effect on the overall hydrogel secondary structure.

Table 4.9. Percentage of secondary structures in hydrogels and magnetogels according to the general range reported in [28-32], obtained through curve-fitting of the amide I band (AI), amide I second derivative (ASD) and amide III band (AIII) of the hydrogelator Npx-L-Lys-Z Δ Phe-OH. Legend: OC: ordered structures (α -helix and β -sheet); UC: unordered structures (turn and random coil).

System	H			CS			D		
Method	AI	ASD	AIII	AI	ASD	AIII	AI	ASD	AIII
α -helix	0	0	7.6	0	0	8.6	0	0	5.0
β -sheet	33.3	23.8	24.0	27.0	15.0	16.9	33.4	23.8	12.4
Turn	27.8	17.7	15.3	33.1	29.3	19.8	29.6	17.2	25.0
Random coil	38.9	58.5	60.7	39.9	55.7	54.7	38.4	59.0	62.6
OC	33.3	23.8	29.4	27.0	15.0	25.4	33.4	23.8	16.6
UC	66.7	76.2	70.6	73.0	85.0	74.6	68.0	76.2	83.4
χ^2	2×10^{-7}	1×10^{-9}	4×10^{-11}	2×10^{-7}	2×10^{-11}	1×10^{-10}	7×10^{-8}	3×10^{-11}	8×10^{-11}

4.4.1.2. Circular dichroism

As discussed in section §2.1.6, a molecule is optically active in circular dichroism (CD) if the chromophore contains an asymmetric (chiral) environment or display an induced chirality owing to three-dimensional bonding structures [33-35]. Hereby, the repetitive and ordered peptide secondary structures lead to characteristic circular dichroism signals, resulting in a spectrum that corresponds to the sum of its conformational elements [35]. The β -sheet structures are characterized by a sharp positive band in the region 195-210 nm and a broad negative band in the region 215-230 nm, while α -helices display negative ellipticity at 222 nm and 208 nm, and positive ellipticity at 193 nm [35]. The random coil structures have a weak positive band at 217 nm and a large negative near 200 nm [35]. These bands are associated with the peptide bond transitions π - π^* and n - π^* , which are centred around 190 nm and 220 nm, respectively [3,36]. The obtained CD spectra of diluted hydrogelator solutions, with and without

nanoparticles, are represented in figure 4.31. The hydrogelator Npx-L-Met-Z Δ Phe-OH solutions show similar spectral shape, where an increase of ellipticity is observed in the presence of core/shell nanoparticles and a red-shift for the magnetogel containing gold-decorated nanoparticles. Overall, the exciton couplets are evidenced by a negative cotton effect with a broad negative ellipticity centred at 220 nm and a slow ellipticity decrease for lower wavelengths, which suggest a predominance of β -sheets and that hydrogelator molecules establish similar intermolecular interactions in hydrogel and magnetogels. Nevertheless, the naphthalene moiety commonly shows exciton couplets between 200 and 230 nm, besides being stronger in magnitude than the peptidic transitions [37]. Thus, the broad negative ellipticity results from the sum of contributions of β -sheets and naphthalene exciton couplets.

The hydrogelator Npx-L-Lys-Z Δ Phe-OH shows a negative cotton effect with the negative ellipticity centred at 230 nm. The high intensity of the negative ellipticity indicates high chiral ordering, which might be associated with the organized chiral stacking of the naphthalene moieties [37]. Moreover, the low ellipticity in the region 190-210 nm of the peptide amide transition suggest that the naphthalene stacking is crucial for the hydrogelator Npx-L-Lys-Z Δ Phe-OH self-assembly, besides the peptide backbone participation on the fibre formation. The sign of the naphthalene exciton couplet indicates the helicity handedness of the chiral arrangement, where naphthalene shows in both hydrogelators a left-handed helicity, thus indicating that the naproxen moieties are highly organized through chiral stacking. Overall, the presence of nanoparticles suggests an influence on the chiral ordering of the assembled structure, though it does not allow any conclusion on the increase of random coil structures comparatively to FTIR results.

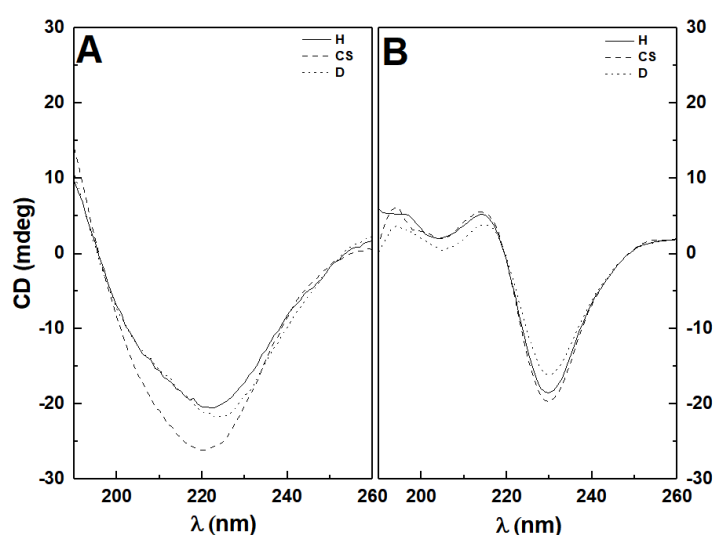


Figure 4.31. Circular dichroism spectra of hydrogel (H), core/shell (10 m/m%) (CS) and gold-decorated manganese ferrite nanoparticles (10 m/m%) (D) based magnetogels circular dichroism spectra of the hydrogelator (A) Npx-L-Met-Z Δ Phe-OH (0.02 wt%, 2 v/v% 1 M NaOH, 0.5 wt% GdL) and (B) Npx-L-Lys-Z Δ Phe-OH (0.02 wt%, pH=13).

4.4.1.3. Transmission Electron Microscopy (TEM)

The transmission electron microscopy (TEM) images of the magnetic nanoparticles are presented in figure 4.32. Core/shell manganese ferrite/gold nanoparticles show an average size of 49.2 ± 18.1 nm, while the gold-decorated manganese ferrite nanoparticles show flocks of 54.0 ± 12.8 nm containing gold nanoparticles with 3.8 ± 0.9 nm. The electron dispersive X-ray (EDX) spectra evidence the presence of gold in both types of nanoparticles (figures A3 and A4 in annex). The nanoparticles work as template surfaces for the growth of hydrogel fibres, which results in a porous structure with an average pore size of 82.2 ± 44.6 nm, where the smallest pore is 20 nm and the largest is 223 nm. The fibres have an average cross section of 11.2 ± 3.6 nm, the thinnest being 5.6 nm and the thickest 21 nm. The high density of nanoparticles and fibre crosslinks suggest that the gels are highly saturated at 10 m/m% of nanoparticles.

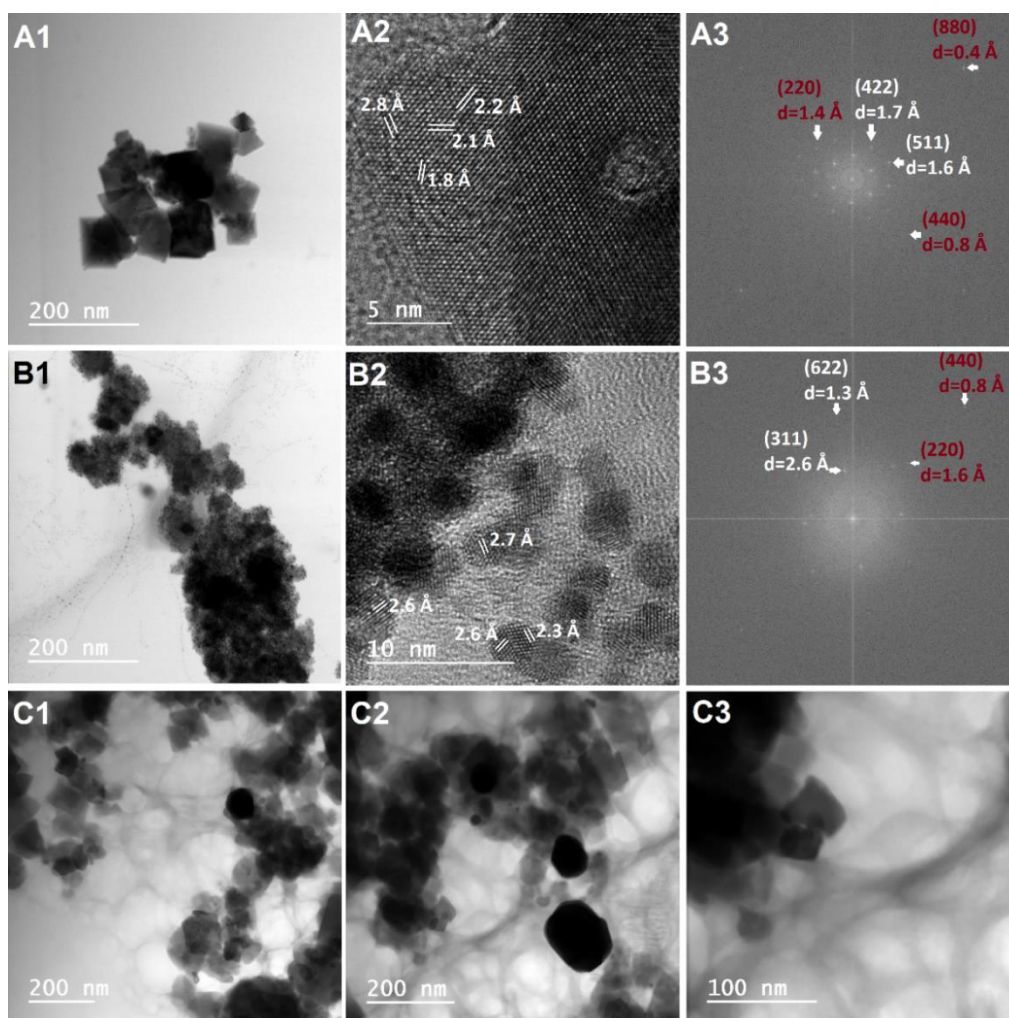


Figure 4.32. Transmission electron microscopy images of (A) core/shell manganese ferrite/gold nanoparticles, (B) gold-decorated manganese ferrite nanoparticles and (C) Npx-L-Met-ZΔPhe-OH (0.08 wt%) magnetogel containing 10 m/m% core/shell manganese ferrite/gold nanoparticles. A3 and B3 are the Fast Fourier Transform of the respective nanoparticles, where some gold planes (red) and manganese ferrite (white) were indexed.

4.4.2. Evaluation of magnetogels as nanocarriers

4.4.2.1. Incorporation of curcumin into hydrogels/magnetogels

The incorporation of curcumin into the hydrogel/magnetogel matrixes required previous evaluation of the spectral overlap of the hydrogelator aromatic groups emission and curcumin absorption (figure 4.33). The absorption and emission spectral overlap of the fluorophores suggests that Förster resonance energy transfer (FRET) is possible, which allows following the incorporation of curcumin into the hydrogels/magnetogels. Moreover, as curcumin is barely soluble in water [38], the resulting fluorescence emission will be an indicator of the presence of curcumin in the hydrophobic cavities or near the fibres.

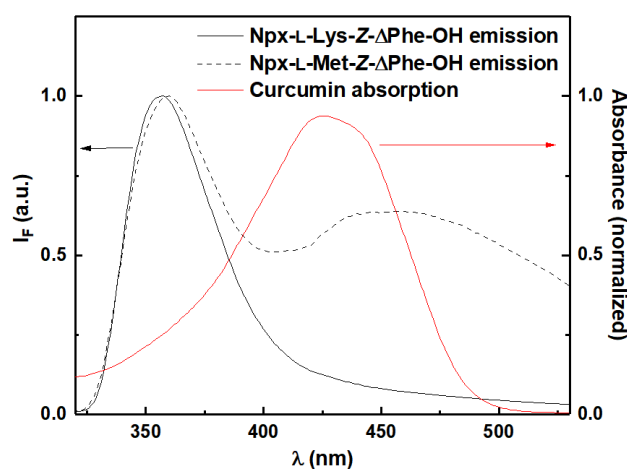


Figure 4.33. Normalized fluorescence emission spectra of hydrogels superposed to curcumin normalized absorption spectrum.

The fluorescence emission of curcumin due to FRET from the hydrogelator Npx-L-Met-Z-ΔPhe-OH around 500 nm is overlapped by the aggregates emission band. However, excitation at 420 nm results in strong emission from curcumin in all hydrogels and magnetogels (figure 4.34), indicating that curcumin is mainly located in hydrophobic domains of the systems network, as it does not emit fluorescence in aqueous environments. Interestingly, for the same nanoparticle mass percentage (10 m/m%) relatively to the hydrogelator and equal molarity of curcumin, highly similar quenching effect of the monomer band was obtained, and similar curcumin to monomer emission ratio was also verified. This demonstrates that the nanoparticles did not significantly affect the distance of curcumin relatively to the fibrils, as the quenching effect on hydrogels and magnetogels due to FRET from the hydrogelator aromatic moieties to curcumin remains similar. Moreover, it is observed that, for the three systems, curcumin emits fluorescence with different bandwidth, shape and wavelength maximum. The magnetogel containing gold-decorated nanoparticles seems to have the stricter variety of environments for curcumin location, owing to its sharper emission band and blue-shifted fluorescence maximum (figure 4.34C). Thus, in this system,

curcumin is restricted to more hydrophobic environments than in the magnetogel based on core/shell nanoparticles and in the hydrogel. Such hydrophobic environments might be associated with the long chain functional groups at the surface of the nanoparticles or changes on the fibres organization due to nanoparticle-fibre interactions.

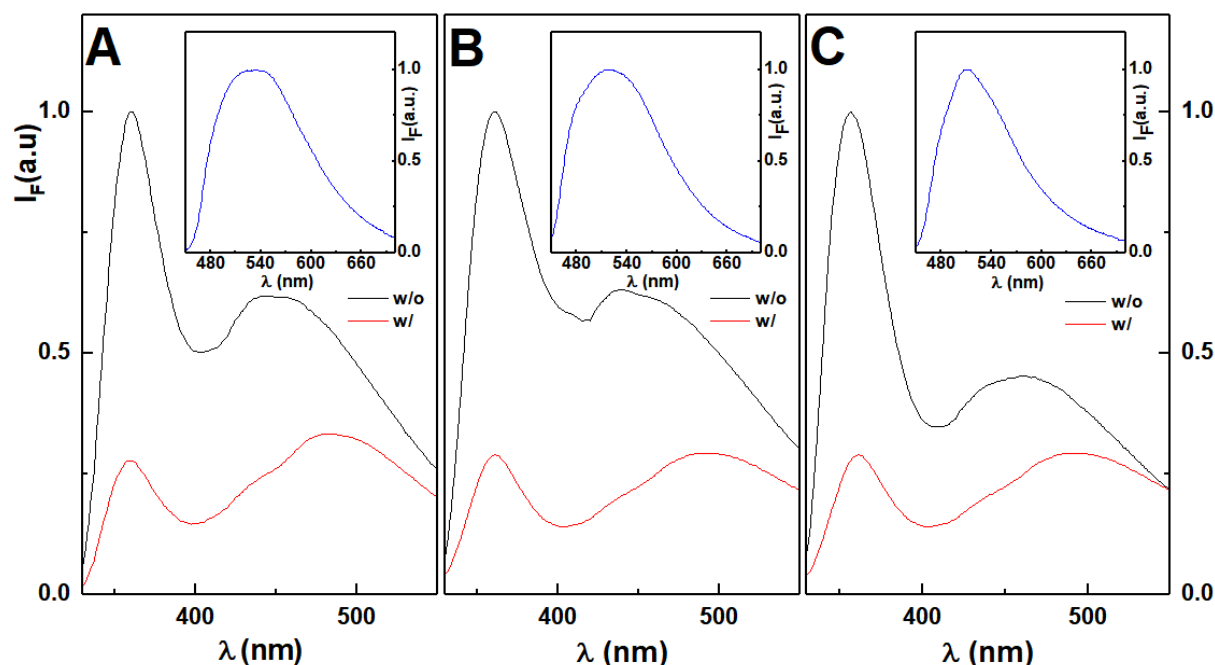


Figure 4.34. Fluorescence emission spectra of Npx-L-Met-Z Δ Phe-OH based magnetogels (1 wt%, 2 v/v% 1 M NaOH, 0.5 wt% GdL), with (w/) and without (w/o) curcumin. (A) Hydrogel, (B) magnetogel with core/shell nanoparticles (10 m/m%), and (C) magnetogel with gold-decorated manganese ferrite nanoparticles (10 m/m%). Insets: Fluorescence emission spectra of curcumin at 2×10^{-6} M ($\lambda_{exc} = 420$ nm).

The fluorescence emission of curcumin due to FRET from the hydrogelator Npx-L-Lys-Z Δ Phe-OH is not clear (figure 4.35). Nevertheless, excitation at 420 nm results in fluorescence emission from curcumin in the hydrogel and magnetogels, therefore suggesting that curcumin is located near the gel fibres, which is further supported by the monomer fluorescence emission quenching of the hydrogel/magnetogel.

The high sensitivity of curcumin to the environment reveals that it is in a slightly more hydrophilic region in the hydrogel compared to the magnetogels, where the strongest interaction might be established with the gold-decorated manganese ferrite nanoparticles owing to the surface functional groups. Moreover, curcumin environment in Npx-L-Lys-Z Δ Phe-OH systems is more polar than in Npx-L-Met-Z Δ Phe-OH ones, as fluorescence spectra are red-shifted in the former, pointing also to different fluorophore environments in Npx-L-Lys-Z Δ Phe-OH based magnetogels (large emission bands).

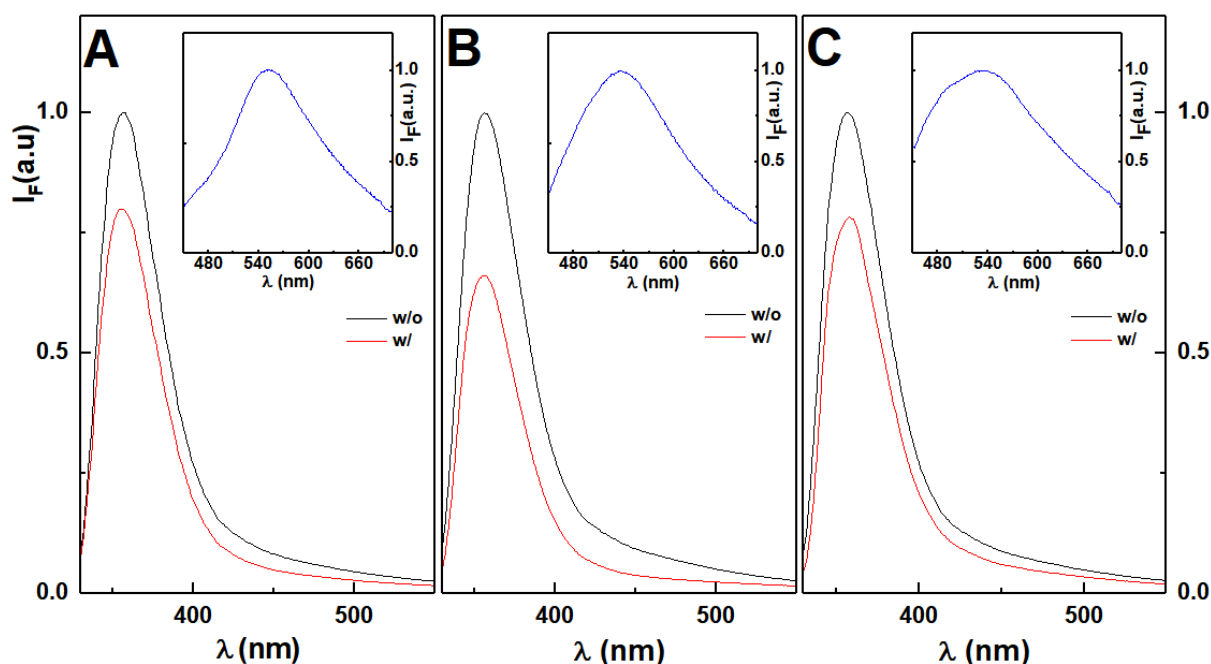


Figure 4.35. Fluorescence emission spectra of Npx-L-Lys-Z Δ Phe-OH based magnetogels (1 wt%, pH=13) with (w/) and without (w/o) curcumin: (A) Hydrogel, (B) magnetogel with core/shell nanoparticles (10 m/m%), and (C) magnetogel with gold-decorated manganese ferrite nanoparticles (10 m/m%). Insets: Fluorescence emission spectra of curcumin at 2×10^{-6} M ($\lambda_{exc} = 420$ nm).

Fluorescence anisotropy values of curcumin (table 4.10) obtained for Npx-L-Met-Z Δ Phe-OH systems demonstrate that the introduction of nanoparticles produced an increase on the microfluidity of the environment where curcumin is located, which can be associated with a different arrangement in the fibres promoted by the nanoparticles, especially in the system containing the core-shell ones. On the other hand, in the systems based on Npx-L-Lys-Z Δ Phe-OH, the reduced fluorescence anisotropy correlates with the hydrophilic (and more fluid) environment felt by curcumin, as previously inferred from the emission spectra. The fluorescence anisotropy values confirm the higher hydrophobicity of Npx-L-Met-Z Δ Phe-OH compared to Npx-L-Lys-Z Δ Phe-OH, which resulted in a higher affinity of curcumin towards the former, even though it can also be related to more compact fibres and a denser network. Indeed, the hydrogels/magnetogels based on Npx-L-Met-Z Δ Phe-OH are far more stable than the ones made from Npx-L-Lys-Z Δ Phe-OH, as the latter highly depend on the room temperature during cooling (during the self-assembly process), affording a softer hydrogel than the former that is hydrogelated through slow pH decrease.

Table 4.10. Fluorescence anisotropy values for curcumin in the hydrogels and magnetogels.

Compound	Hydrogel	Magnetogel with core/shell NPs	Magnetogel with decorated NPs
Npx-L-Met-ZΔPhe-OH	0.29	0.11	0.25
Npx-L-Lys-ZΔPhe-OH	0.12	0.12	0.19

The fluorescence quantum yields were determined as described in section §3.3.1.4., where values of 0.041 and 0.029 were obtained for the hydrogelator Npx-L-Met-ZΔPhe-OH and Npx-L-Lys-ZΔPhe-OH, respectively. The calculated FRET efficiency (Φ_{FRET}), Förster radius (R_0 (nm)), and donor-acceptor distances (R_{DA} (nm)) are represented in table 4.11.

The systems based on Npx-L-Met-ZΔPhe-OH have donor-acceptor distances in the range of reported values for curcumin incorporated in magnetogels (2.2-2.6 nm) [39], while the Npx-L-Lys-ZΔPhe-OH systems show larger distances.

Table 4.11. Calculated FRET efficiency (Φ_{FRET}), Förster radius (R_0 (nm)), and donor-acceptor distances (R_{DA} (nm)) from the interaction of curcumin with the hydrogels (H), and core/shell (CS) and decorated (D) nanoparticle-based magnetogels.

Nanosystem	Npx-L-Met-ZΔPhe-OH			Npx-L-Lys-ZΔPhe-OH		
	H	CS	D	H	CS	D
Φ_{FRET}	0.72	0.73	0.73	0.21	0.36	0.23
R_0 (nm)	2.87	2.89	2.82	2.64	2.63	2.64
R_{DA} (nm)	2.45	2.44	2.40	3.30	2.90	3.22

The introduction of nanoparticles did not produce a significant change on the distance between the Npx-L-Met-ZΔPhe-OH aromatic moieties and curcumin in all the nanosystems. However, the presence of nanoparticles seems to have increased the curcumin affinity towards the hydrogelator Npx-L-Lys-ZΔPhe-OH, which correlates with the higher anisotropy compared to the hydrogel and the blueshift of curcumin emission due to more hydrophobic environments. Nevertheless, it is clearly noticed that the Npx-L-Lys-ZΔPhe-OH based hydrogel/magnetogels show a larger donor-acceptor distance than in the Npx-L-Met-ZΔPhe-OH.

Overall, the results suggest that both nanoparticles introduce different effects on curcumin location and on the hydrogel structure, which might ultimately change the bulk mechanical properties.

4.4.2.2. Drug delivery to biomembrane models

The potential of the developed magnetogels as drug delivery systems was assessed using the fluorescent dye Nile Red. Nile Red is a solvatochromic probe that intensely emits fluorescence in non-polar environments, but has almost negligible emission in water, the emission in non-polar regions being accompanied by a blueshift with reduction of polarity [40-43]. Its high hydrophobic character leads to its preferential localization in the lipid membranes, which can be explored to follow up the interaction between hydrogels/magnetogels and biomembrane models. Hereby, curcumin was incorporated into the hydrogel/magnetogel and Nile Red into small unilamellar vesicles (SUVs). FRET can occur between these fluorophores, as evidenced by the overlap of the curcumin fluorescence emission and Nile Red absorption band in SUVs (figure 4.36). Although Nile Red can be directly excited at 420 nm, such is highly inefficient, thus the increased Nile Red fluorescence will be a result of the FRET process.

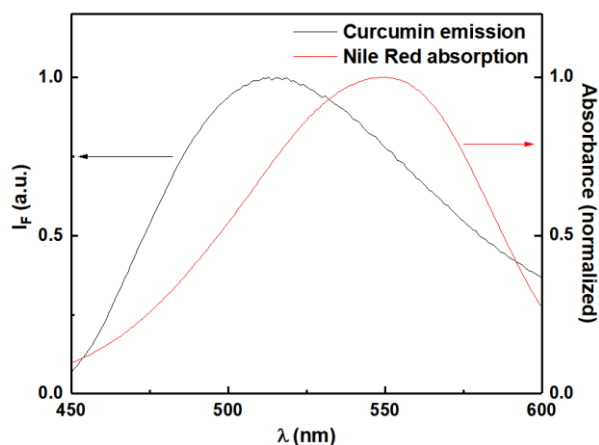


Figure 4.36. Curcumin normalized fluorescence emission spectra superposed to Nile Red normalized absorption spectrum.

The interaction of magnetogels/hydrogels with membrane models can occur either by SUVs penetration into the hydrogel/magnetogel matrix, or through drug delivery. In the latter, the drug incorporated in the hydrogel/magnetogel diffuses to the biomembranes. The used hydrogelators at a concentration of 1 wt% might have small cavities, as no FRET was obtained in the hydrogel/magnetogel matrix, i.e. the biomembranes did not penetrate the matrix. Nevertheless, the aqueous phase containing the added SUVs exhibited curcumin fluorescence emission and FRET to Nile Red, approximately one hour after its addition. The curcumin emission after interaction is compared to the one of curcumin in SUVs, for the same final concentration (1 μ M), in figures 4.37 and 4.38.

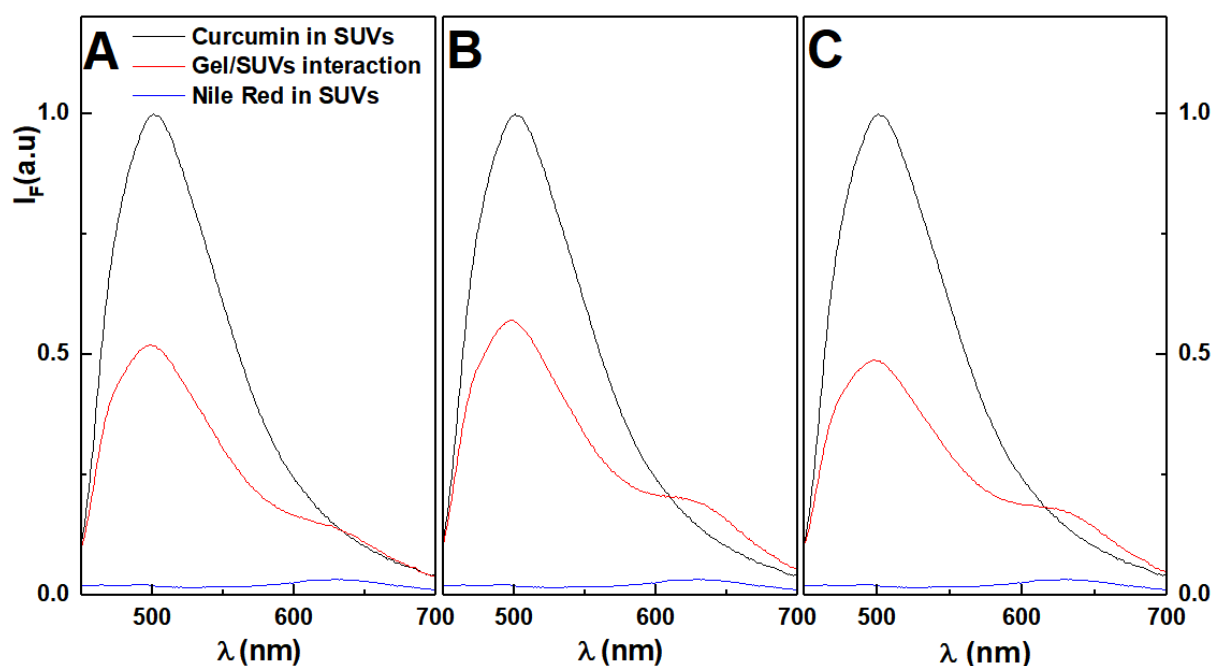


Figure 4.37. Fluorescence emission spectra of SUVs loaded with curcumin (1 μ M), and of curcumin-loaded (2 μ M) (A) hydrogel, (B) core/shell and (C) gold-decorated manganese ferrite nanoparticle-containing magnetogels based on Npx-L-Met-Z Δ Phe-OH after interaction with egg-PC/cholesterol SUVs at 7:3 ratio loaded with Nile Red (2 μ M), through excitation at 420 nm.

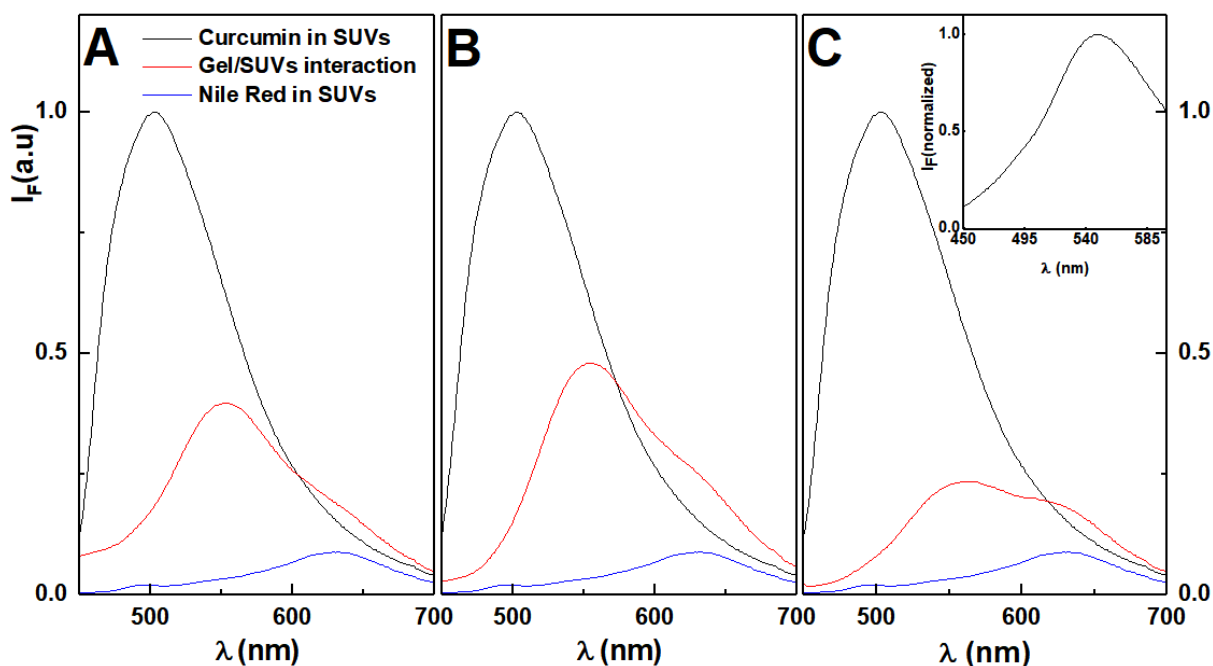


Figure 4.38. Fluorescence emission spectra of SUVs loaded with curcumin (1 μ M), and of curcumin-loaded (2 μ M) (A) hydrogel, (B) core/shell and (C) gold-decorated manganese ferrite nanoparticle-containing magnetogels based on Npx-L-Lys-Z Δ Phe-OH after interaction with egg-PC/cholesterol SUVs at 7:3 ratio loaded with Nile Red (2 μ M), through excitation at 420 nm. Inset: Curcumin fluorescence emission in SUVs at pH=13.

The FRET between curcumin and Nile Red in SUVs after interaction with the gels compared to SUVs containing only curcumin indicates that drug delivery has occurred upon interaction between the

hydrogel/magnetogel (loaded with curcumin) and SUVs (loaded with Nile Red). Moreover, comparison with curcumin inside hydrogel/magnetogel based on Npx-L-Met-Z Δ Phe-OH in section §4.4.2.1. reveals that curcumin has localized to a more hydrophobic environment (biomembranes), as its maximum emission wavelength after interaction is similar to the one of curcumin in SUVs, suggesting that the hydrogel/magnetogel did not interfere with the organization of biomembranes. On the other hand, the hydrogel/magnetogels based on Npx-L-Lys-Z Δ Phe-OH induce a large shift of curcumin fluorescence emission to longer wavelengths, thus indicating that the hydrogelator might have interfered on the stability of biomembranes and/or curcumin was even degraded, as suggested by the variability of curcumin intensities. Considering that Nile Red emission does not suffer any shift after interaction with the hydrogel/magnetogels, then the observed curcumin emission shift may be associated with a curcumin ionized state. To confirm this assumption, SUVs loaded with curcumin were mixed with pH=13 buffer and curcumin emission showed the maximum shifted to 550 nm, therefore confirming that curcumin is ionized after the interaction with the hydrogel/magnetogels. Hereby, although the hydrogel Npx-L-Lys-Z Δ Phe-OH can be prepared without requiring organic solvents, it is demonstrated that it is not adequate for drug delivery owing to the extreme pH conditions required for its preparation, which do not allow a proper stabilization of curcumin, due to its degradation being accelerated at basic pH [44]. Additionally, these requirements are not compatible with the physiological conditions, besides reducing the distribution of curcumin to membranes, as it is more hydrophilic in the deprotonated state.

4.4.3. Evaluation as multimodal therapeutic agents

In this section, the hydrogels/magnetogels based on Npx-L-Met-ZΔPhe-OH were prepared at 0.3 wt%, to have a comparable concentration value relatively to other reported hydrogels and magnetogels [3,39], which demonstrated its promising elastic properties. The systems of Npx-L-Lys-ZΔPhe-OH were kept at 1 wt%, as it is the critical gelation concentration when prepared at pH=13. Moreover, only the gels based on Npx-L-Met-ZΔPhe-OH proceeded to drug release assays, as the gels based on Npx-L-Lys-ZΔPhe-OH showed to be inadequate as drug delivery nanosystems

4.4.3.1. Photo-induced hyperthermia in magnetogels

The irradiation assay in curcumin-loaded magnetogels was carried out using a 200 W xenon arc lamp and a cut-on wavelength at 600 nm. In this experiment, curcumin fluorescence was measured for 4 h. Once the irradiation period ended, the lamp was turned off, and fluorescence was measured and repeated after one hour. Upon irradiation, the surface plasmon resonance not only enhances the local electromagnetic field that leads to an increase of fluorescence quantum yield, but also allows the conversion of the absorbed light into heat, through nonradiative processes that are associated with the fast phase loss of the coherently excited electrons [45,46]. The heat generated by the nanoparticles will be transferred to the medium, which is accompanied by the formation of vapor nanobubbles, where the expansion occurs until cooling and the collapse leads to a stress wave that propagates in the surrounding medium [47]. Hereby, curcumin was used as a fluorescence thermometer to determine the mean temperature in the vicinity of the nanoparticles surface when embedded inside the hydrogel matrix.

The non-radiative rate constant (k_{nr}) can be related with temperature through the Arrhenius equation:

$$k_{nr} = k_o e^{-\frac{E_a}{RT}} \quad (4.6)$$

where E_a is the activation energy and k_o is a pre-exponential factor. Considering that the fluorescence quantum yield is related to the radiative and non-radiative rate constants through the equation:

$$\Phi_F = \frac{k_F}{k_F + k_{nr}} \Rightarrow \frac{1}{\Phi_F} - 1 = \frac{k_{nr}}{k_F} \quad (4.7)$$

the fluorescence quantum yield at room temperature (Φ_F^0) can be used as a reference:

$$\frac{\Phi_F^0}{\Phi_F} - \Phi_F^0 = \Phi_F^0 \frac{k_{nr}}{k_F} \quad (4.8)$$

and be related with the fluorescence intensity variation:

$$\frac{I_F^0}{I_F} - \Phi_F^0 = \Phi_F^0 \frac{k_{nr}}{k_F} \quad (4.9)$$

If the fluorescence quantum yield of curcumin close to the nanoparticles is negligible, then the dependence of fluorescence intensity (I_F) on temperature can be expressed as:

$$\frac{I_F^0}{I_F} \propto \Phi_F^0 \frac{k_0}{k_F} e^{-\frac{E_a}{RT}} \Rightarrow \frac{I_F^0}{I_F} \propto A e^{-\frac{E_a}{RT}} \quad (4.10)$$

where Φ_F^0 and I_F^0 are, respectively, the quantum yield and fluorescence intensity at room temperature without irradiation, and E_a is the activation energy. The representation of $\ln(\frac{I_F^0}{I_F})$ as a function of $1/T$ will be linear. Curcumin showed a slope of -60.966 K through a linear regression when temperature was changed from 20 °C to 60 °C (figure 4.39).

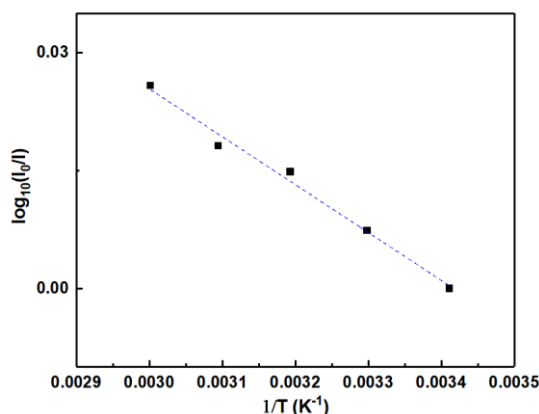


Figure 4.39. Normalized fluorescence variation of curcumin at increasing temperature, 0.3 wt% of the Npx-L-Met-ZΔPhe-OH hydrogelator and 4 μM of curcumin, with an $R^2 = 0.989$ and $\log_{10}\left(\frac{I_0}{I}\right) = -60.966\left(\frac{1}{T}\right) + 0.2083$.

The obtained logarithm of the variation of fluorescence intensity of curcumin in the magnetogels subjected to continuous irradiation ($\lambda > 600$ nm) allowed extrapolation of the nanoparticles surface temperature through the above determined parameter. The results demonstrate that a heating effect is being produced upon continuous irradiation of the magnetogels (figure 4.40), and when the irradiation is stopped a temperature decrease (fluorescence increase) is observed, thus suggesting that the heating effect is reversible. Hereby, it is expected that irradiation will enhance drug release from the magnetogel to the medium.

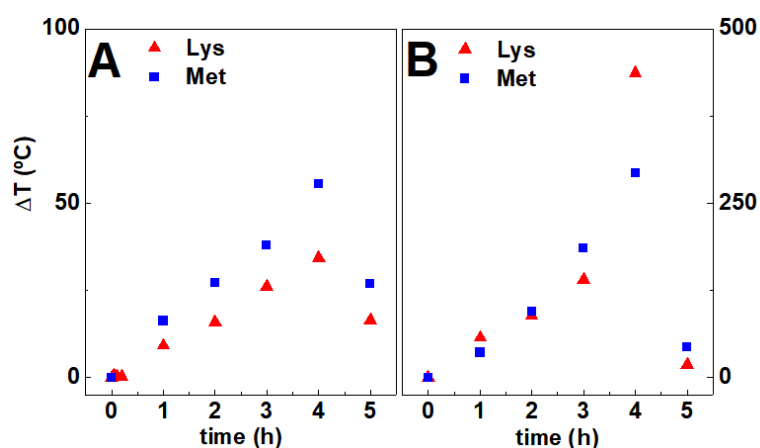


Figure 4.40. Temperature variation through irradiation of (A) core/shell nanoparticles and (B) gold-decorated manganese ferrite nanoparticles in the Npx-L-Met-ZΔPhe-OH at 0.3 wt% (Met) and Npx-L-Lys-ZΔPhe-OH at 1 wt% (Lys) magnetogels at 10 m/m% of nanoparticles.

The exponential temperature increase obtained for the gold-decorated nanoparticles containing magnetogels might be associated with the thermal degradation of curcumin, besides the increased non-radiative relaxation rate by the heating of gold nanoparticles. The thermal degradation of curcumin results in the formation of vanillin, ferulic acid and 4-vinyl guaiacol, that have biological activity (antioxidant and anti-inflammatory) [48]. Nevertheless, the observed temperature decrease (fluorescence recovery) when irradiation is turned off suggests that, even if degradation is being accelerated (which reduces fluorescence emission and increases the estimated temperature variation), its impact is not enough to explain the high temperature increase produced by the nanoparticles. Therefore, a possible hypothesis is that the high gold nanoparticle concentration and the associated surface functionalization might favour a larger accumulation of curcumin around the nanoparticle cluster and higher temperatures, as the heat will have greater difficulty to dissipate than in the core/shell nanoparticles that are in direct contact with water.

4.4.3.2. Photo-induced drug release

The release of curcumin from the hydrogels and magnetogels were evaluated with and without excitation of nanoparticles plasmon band. The nanosystems show an initial burst release, followed by a slower release phase, where a similar behaviour was observed either in hydrogels and irradiated magnetogels on the first 8 hours. The obtained results for plasmonic magnetogels was also compared to magnetogels containing manganese ferrite nanoparticles to assess the influence of the gold shell on drug release. An interaction between curcumin and gold-containing nanoparticles is also evidenced by the drug release

profiles (figure 4.41 and figure A5), as a lower curcumin concentration and drug release kinetics is observed on the non-irradiated magnetogels, while a higher release was obtained for the manganese ferrite containing magnetogels.

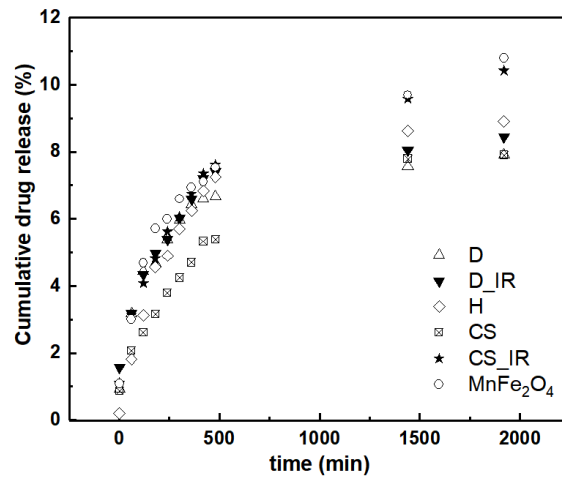


Figure 4.41. Cumulative drug release profiles of curcumin (20 μ M) in 0.3 wt% Npx-L-Met-Z Δ Phe-OH hydrogel (H), and magnetogels containing 10 m/m% core/shell (CS) or decorated (D) nanoparticles with and without incidence of radiation (IR) and MnFe₂O₄ nanoparticles.

Therefore, the presence of manganese ferrite nanoparticles suggests that the inclusion of nanoparticles is expected to open the hydrogel pores and facilitate drug release, while nanoparticles capable of interacting with the incorporated drug will difficult its release. This effect is more pronounced on gold-decorated nanoparticles, where the surface functionalization might have hindered the release of curcumin.

To quantitatively evaluate the effect of nanoparticles and irradiation on the magnetogel, several mathematical models were adjusted to the cumulative drug release profiles. The first-order model is usually applied to the drug dissolution of water-soluble drugs in porous matrices and expressed as [49,50]:

$$C_t = C_0 e^{kt} \quad (4.11)$$

where C_t and C_0 are the remaining concentration at time t and $t=0$, and k is the dissolution rate constant. The Hixson-Crowell model describes the remaining concentration at time t from a system that changes in surface area and diameter over time, but the initial geometrical form is kept constant [49]:

$$C_t^3 = C_0^3 - kt \quad (4.12)$$

where k integrates the surface-volume relation changes over time. The Korsmeyer-Peppas model is applicable to polymeric systems that include both diffusion and erosion of polymer chains, which is

described by a rate constant K_s that include the structural and geometrical characteristics of the nanosystem and a coefficient n that is related with the diffusion mechanism [49-52]:

$$C_t = C_0 K_s t^n \quad (4.13)$$

When $n < 0.45$, the release mechanism is diffusion-controlled (Fickian release), $0.45 < n < 0.89$ indicates a combination of diffusion and erosion drug release (non-Fickian release), $0.89 < n < 1$ is a relaxation-controlled release, and if $n > 1$, then the release is controlled by swelling and polymer chain relaxation [49-52].

The Higuchi model assumes that the drug concentration in the nanosystem is much higher than its solubility, drug diffusion occurs in a single dimension and diffusivity is constant, the drug particle size is much smaller than the nanosystem thickness, effects due to matrix swelling and dissolution are negligible, and that the release environment provides perfect sink conditions, i.e. the solvent volume is much greater than the saturated nanosystem solution [48,50]. A simplified Higuchi model can be described according to the equation [49,51]:

$$C_t = C_0 K_H \sqrt{t} \quad (4.14)$$

where K_H represents the Higuchi dissolution constant.

The Gompertz model adequately describes the release profiles where the drug has good solubility and intermediate release rates according to the equation [49]:

$$X_t = X_{max} e^{-ae^{b \log_{10} t}} \quad (4.15)$$

where X_t and X_{max} is the dissolved fraction percentage at time t and its maximum, a is a shape parameter and b is the dissolution rate per unit of time.

The coefficients of determination of each model obtained for curcumin release on the nanosystems based on the hydrogelator Npx-L-Met-ZΔPhe-OH, with and without irradiation are shown in table 4.12.

Table 4.12. Coefficients of determination (R^2) obtained for the release of curcumin in the nanosystems based on Npx-L-Met-Z-ΔPhe-OH.

	First-order	Hixson-Crowell	Higuchi	Korsmeyer-Peppas	Gompertz
Hydrogel	-0.24	-0.38	0.76	0.92	0.99
CS	-0.36	-0.10	0.90	0.98	0.99
CS_IR	-0.36	-0.62	0.77	0.90	0.99
D	-0.36	-2.06	0.29	0.95	0.99
D_IR	-0.36	-2.07	0.01	0.93	0.98
MnFe ₂ O ₄	-0.36	-0.77	0.72	0.98	0.99

Overall, R^2 values demonstrate that the results are not modelled by the first-order and the Hixson-Crowell model, i.e. the released drug (curcumin) is not water soluble and the hydrogel does not change in diameter overtime. The results are best fitted by a Gompertz model, but the Korsmeyer-Peppas model also suggests that the release can be associated to a diffusion-controlled mechanism. Hereby, the determined kinetic dissolution constants and parameters of these latter models are represented in table 4.13. The Gompertz model parameters demonstrate that the presence of nanoparticles hampered the release rate of curcumin comparatively to the hydrogel, where the core/shell nanoparticles produced the steepest difference. This effect can be associated with either an interaction between curcumin and the nanoparticles, or the nanoparticles introducing inhomogeneities in the hydrogel matrix that difficult the drug diffusion through it. Moreover, the irradiated magnetogels demonstrated a higher drug release kinetics, where the highest b coefficient increase is observed for the core/shell containing magnetogel or the control with manganese ferrite nanoparticles. On the other hand, the lower b coefficient increase of the gold-decorated nanoparticles can be associated with the surface functionalization not favouring the release of curcumin, even if the nanoparticles attain higher temperature compared to the core/shell ones, as previously observed in section §4.4.3.1. Although the irradiation does not significantly change the maximum dissolution, the nanoparticles show an impact on the predicted maximum curcumin dissolution. Such can be associated with a perturbation of free energy difference between the magnetogel matrix and the solution, i.e. the core/shell nanoparticles might induce larger pores that leads to a reduced affinity of curcumin towards the matrix while the decorated nanoparticles due to the functionalized surface might lead to a higher affinity of the hydrophobic drug towards the matrix.

Table 4.13. Release coefficients of the Korsmeyer-Peppas and Gompertz models obtained for the curcumin release profiles in the nanosystems based on Npx-L-Met-ZΔPhe-OH.

	Korsmeyer-Peppas		Gompertz		
	Ks	n	X_{max}	a	b
Hydrogel	0.87	0.32	10.48	36.97	1.67
CS	0.51	0.37	18.89	8.11	0.69
CS_IR	1.99	0.21	18.89	7.25	0.76
D	3.93	0.21	9.25	11.23	1.30
D_IR	3.72	0.23	9.99	13.27	1.34
MnFe ₂ O ₄	1.18	0.29	18.96	6.54	0.73

Considering the potential anti-inflammatory activity of the hydrogelator, release profiles were also assessed in all nanosystems with and without irradiation (figure 4.42 and figure A6). The profiles suggest that the nanoparticles did not severely affected the release of the hydrogelator, with the highest accumulation percentage being attained on the core/shell nanoparticles containing magnetogel with irradiation. Moreover, the profile shape tendency reveals that the hydrogelator release tends to a saturation as also observed for curcumin. Therefore, after hydrogelation a certain amount of free hydrogelator is in equilibrium with the aggregate state in the hydrogel matrix, which is released to the buffer when added, but if a saturation is attained such can be due to a limit of solubility of the hydrogelator or an equilibrium between the free hydrogelator in buffer and in the hydrogel matrix, and/or an equilibrium between the hydrogelator in aggregate state incorporating the hydrogel fibres and the free hydrogelator. However, the first hypothesis means that the hydrogelator would precipitate, while the combined second and third hypotheses suggest that an erosion/degradation of the hydrogel fibres occur, but the amount of added buffer is insufficient to drive the hydrogel dissolution.

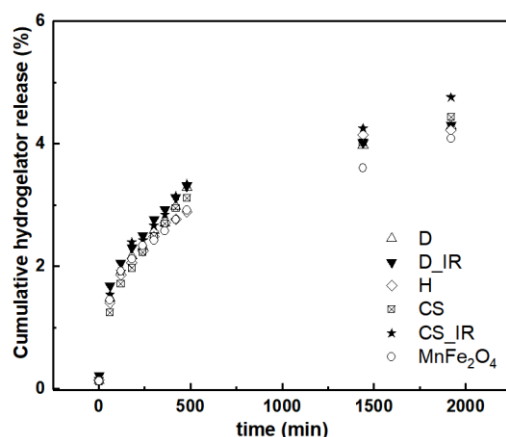


Figure 4.42. Cumulative drug release profiles of the hydrogelator in 0.3 wt% Npx-L-Met-Z Δ Phe-OH hydrogel (H), and magnetogels containing 10 m/m% core/shell (CS) or decorated (D) nanoparticles with and without incidence of radiation (IR) and MnFe₂O₄ nanoparticles.

The release profiles were fitted with the previous described drug release models (table 4.14), showing that it cannot be described by the dissolution of a water-soluble drug or geometrical changes of the hydrogel or magnetogel. The good fitting of the other models suggest that the release mechanism can be described by a diffusion-controlled mechanism.

Table 4.14. Coefficients of determination (R^2) obtained for the release of hydrogelator in the nanosystems based on Npx-L-Met-Z Δ Phe-OH.

	First-order	Hixson-Crowell	Higuchi	Korsmeyer-Peppas	Gompertz
Hydrogel	-0.24	-0.49	0.80	0.99	0.99
CS	-0.31	-0.31	0.84	0.99	0.99
CS_IR	-0.29	-0.51	0.80	0.99	0.99
D	-0.50	-0.72	0.73	0.99	0.99
D_IR	-0.74	-1.12	0.61	0.99	0.99
MnFe ₂ O ₄	-0.89	-0.89	0.68	0.99	0.99

However, the nanoparticles only show a small impact on the hydrogelator release profile compared to the hydrogel, which can be associated with the 10 m/m% content of nanoparticles not being enough to severely affect the dissolution of the hydrogel matrix along time. The Korsmeyer-Peppas model suggest that the irradiation contributed to an acceleration of the hydrogelator release, i.e. favoured the release of hydrogelator monomers from the matrix fibre aggregates. Nevertheless, the lack of agreement with the Gompertz model and the small difference on the release profiles does not allow a proper conclusion on the effect of the nanoparticles and irradiation on the acceleration of the hydrogelator release rate.

Table 4.15. Release coefficients of the Korsmeyer-Peppas and Gompertz models obtained for the hydrogelator release profiles in the nanosystems based on Npx-L-Met-Z Δ Phe-OH.

	Korsmeyer-Peppas		Gompertz		
	Ks	n	X_{max}	a	b
Hydrogel	0.42	0.3094	32.93	5.37	0.296
CS	0.38	0.3291	10.51	6.47	0.611
CS_IR	0.47	0.3079	24.63	5.22	0.350
D	0.50	0.2875	11.01	5.15	0.517
D_IR	0.62	0.2580	10.19	4.55	0.507
MnFe ₂ O ₄	0.52	0.2710	21.43	4.64	0.309

The irradiation influence on the release of curcumin to biomembrane models was assessed by measuring the interaction during 8 h. The hydrogel and magnetogels without irradiation show a similar sigmoidal behaviour (figure 4.43), while the magnetogels subjected to irradiation show an accelerated accumulation of curcumin in the biomembrane models in the first two hours. Hereby, considering that the saturation is due to curcumin attaining a partitioning equilibrium between the hydrogel/magnetogel and the limited number of available liposomes, the initial release acceleration that was also observed in the release to buffer further demonstrates that photothermia might be used as a trigger to burst the release of the encapsulated drug.

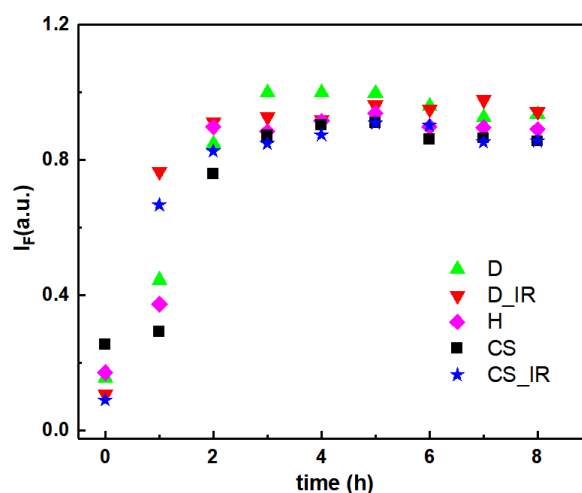


Figure 4.43. Maximum fluorescence emission intensity of curcumin in egg-PC/cholesterol SUVs at 7:3 ratio, through excitation at 420 nm, during the interaction with the Npx-L-Met-Z Δ Phe-OH hydrogel (H), core/shell (CS) and gold-decorated manganese ferrite (D) nanoparticle-containing magnetogels with and without irradiation (IR) loaded with curcumin (20 μ M). The hydrogelator is at a concentration of 0.3 wt% and 10 m/m% of nanoparticles.

4.4.3.3. Magnetic properties

The magnetic properties can be determined from the magnetic hysteresis loop (figure 4.44), which relates the induced magnetic moment with the applied magnetic field (H). Considering that the here described core/shell manganese ferrite/gold nanoparticles show similar core (13 nm) and shell (5 nm) sizes to previously reported nanoparticles in reference [21], they are expected to have also similar magnetic properties. Therefore, the magnetic properties were only determined for the gold-decorated manganese ferrite nanoparticles and are compared to the already reported values (table 4.16).

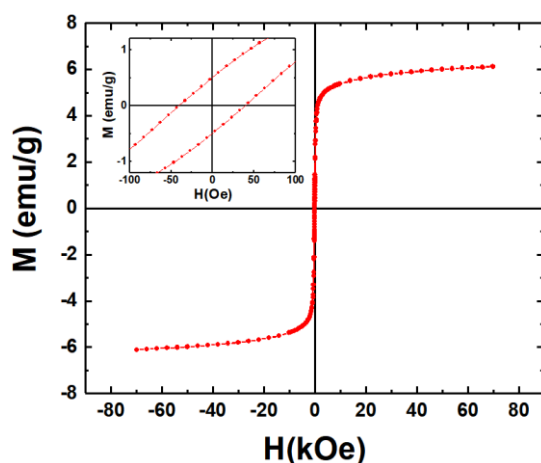


Figure 4.44. Magnetization hysteresis loop of gold-decorated manganese ferrite nanoparticles measured at room temperature. Inset: Enlargement of the loop in the low field region.

Table 4.16. Coercive field (H_c), saturation magnetization (M_s), remnant magnetization (M_r), and ratio M_r/M_s for core/shell manganese ferrite/gold nanoparticles (CS) reported in reference [21], and gold-decorated manganese ferrite nanoparticles (D) at room temperature.

	H_c (Oe)	M_s (emu/g)	M_r (emu/g)	M_r/M_s
CS	13.57	3.15	0.08	0.03
D	39.1	6.13	0.52	0.08

The gold-decorated nanoparticles show similar magnetic properties compared to the core/shell manganese ferrite/gold nanoparticles, as evidenced from the M_r/M_s ratio, which is smaller than 0.1 in both nanoparticles, i.e. the nanoparticles are superparamagnetic [21]. Nevertheless, the saturation magnetization is higher owing to a lower diamagnetic contribution from the MPA-coated gold nanoparticles shell compared to the bulk gold shell.

Therefore, both nanoparticles design shows promising results for magnetic targeting and hyperthermia, though the gold-decorated manganese ferrite nanoparticles design represents an improvement due to the reduction of the diamagnetic contribution.

4.4.3.4. Rheological properties

The mechanical spectra of equilibrated Npx-L-Met-Z Δ Phe-OH hydrogel and magnetogels (10, 20 and 30 m/m%) are shown in figure 4.45. The rheological data measured for magnetogels formulated with 10 m/m% nanoparticles underline the effects of the nanoparticles chemical modification on hydrogel elasticity. The addition of gold-decorated manganese ferrite nanoparticles results in the reinforcement of the hydrogel elasticity, as G' is five times larger (figure 4.45A and table 4.17). In contrast to this, the addition of 10 m/m% core/shell nanoparticles leads to a significant reduction (10 times) of the gel elasticity (figure 4.45B).

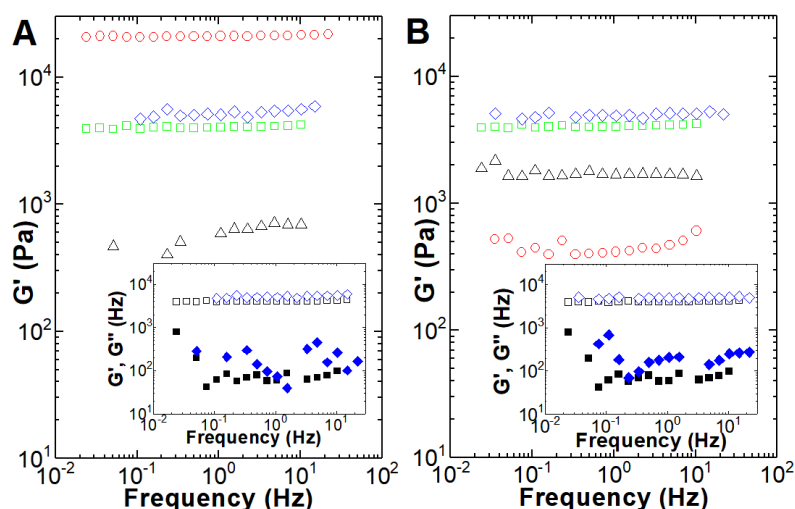


Figure 4.45. Frequency dependence of the shear elastic modulus G' for hydrogels formulated with 0 m/m% (squares), 10 m/m% (circles), 20 m/m% (triangles) and 30 m/m% (diamonds) of gold-decorated nanoparticles (A) or core/shell nanoparticles (B). Insets: mechanical spectra (G' , empty symbols; G'' , solid symbols) of the hydrogel (squares) and of the magnetogels formulated with 30 m/m% nanoparticles (diamonds).

Table 4.17. Rheologic properties of hydrogel and magnetogels containing core/shell manganese ferrite/gold (CS) and gold-decorated manganese ferrite (D) nanoparticles. The critical strain percentage is taken at the breaking point.

	Dynamic frequency sweep		Dynamic strain sweep		
	G' (Pa)	G'' (Pa)	G' (Pa)	G'' (Pa)	Critical Strain (%)
Hydrogel	4175	80.5	4670	136	1.5
CS 10 m/m%	501.5	49.2	-	-	2.1
CS 20 m/m%	1895	55.2	-	-	1.6
CS 30 m/m%	4975	172.8	5125	97.05	1.3
D 10 m/m%	23850	278.5	-	-	2.1
D 20 m/m%	600	31.9	-	-	1.4
D 30 m/m%	5315	180.3	5485	138	1.1

Depending on the function and location of the tissue, the elastic modulus of native tissues ranges from 0.1 kPa (brain) to 100 kPa (cartilage) [53]. The developed hydrogel and magnetogels elastic modulus fall in the range 1 kPa to 100kPa, which covers soft tissues such as skin, pancreas, spleen, glands and muscles [53]. Therefore, from a mechanical perspective, the hydrogel and magnetogels elastic properties seem to be promising for biomedical applications.

Relatively to previous reported hydrogels with similar chemical nature and prepared at similar concentration, the methionine residue hydrophobic collapse seems to have reinforced the elasticity of the hydrogel (4 kPa at 0.3 wt%) compared to hydrogels containing phenylalanine (Npx-L-Phe-Z Δ Phe-OH, 1.7 kPa at 0.4 wt%), valine (Npx-L-Val-Z Δ Phe-OH, 0.7 kPa at 0.6 wt%) or alanine (Npx-L-Ala-Z Δ Phe-OH, 0.8 kPa at 0.8 wt%), and similar to tryptophan containing hydrogel (Npx-L-Trp-Z Δ Phe-OH, 4 kPa at 0.4 wt%) [3,54]. Thus, a long hydrophobic residue close to the *N*-terminal aromatic moiety is expected to favour a higher elasticity.

The addition of more than 10 m/m% of nanoparticles changes the picture, since the viscoelastic features of the hydrogel are progressively recovered. As a result, adding 30 m/m% nanoparticles does not bring any significant viscoelastic change to the hydrogel. The hydrogel elasticity is only slightly enhanced (the shear elastic modulus G' shifts from 4000 Pa for the hydrogel to 5000 Pa for magnetogel doped with 30% nanoparticles). No differences in the hydrogel structure can be inferred from the frequency dependence of both G' and G'' (mechanical spectra in the insets of figure 4.45), which overall suggest that the linear viscoelastic properties of magnetogels prepared with 30 m/m% nanoparticles are not sensitive to the particle-particle or particle-hydrogelator interactions, in the range of frequencies tested. Similar conclusions were drawn from the mechanical spectra of gels containing smaller contents in nanoparticles (see figure A7 in annex).

Nonlinear viscoelastic data were recently shown to be efficient in revealing the hierarchical structures of gelatin gels [55]. Therefore, large amplitude oscillatory shear strain sweeps (LAOSS) were performed in an attempt to assess possible structural differences in gels prepared with the two types of particles. The strain dependence of the ratio G'/G_0 (G_0 being the value of the shear modulus G' measured at 1Hz in the linear viscoelastic regime) is presented in figure 4.46.

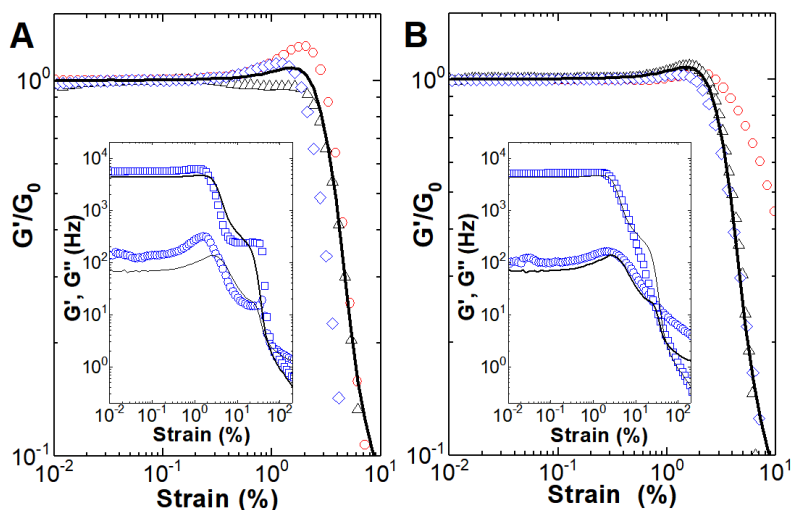


Figure 4.46. Strain dependence of the shear elastic modulus G' scaled by its value (G_0) measured in the linear regime for hydrogels formulated with gold-decorated nanoparticles (A) and with core/shell nanoparticles (B): 0 m/m% (lines), 10 m/m% (circles), 20 m/m% (triangles) and 30 m/m% (diamonds). Inset: strain dependence of the elastic (G' , squares) and loss (G'' , circles) moduli for hydrogels formulated with 0 m/m% (lines) and 30 m/m% (symbols) of nanoparticles.

Overall, the strain dependence of the ratio G'/G_0 indicates a strain hardening behavior for all samples but the gel formulated with 20 m/m% gold-decorated manganese ferrite nanoparticles. Strain hardening, characterized by an increase in G' with the strain, is the nonlinear mechanical signature of a wide range of gels [56] with structures ranging from networks of semi flexible filaments [57] to networks of strongly linked fractal flocs of colloidal particles [58]. Magnetogels formulated with the gold-decorated manganese ferrite nanoparticles are more sensitive to shear deformation, since the critical strain beyond which the strain hardening kicks in is smaller (0.2% against 0.4%). In addition, these magnetogels exhibit a more intense hardening, as the maxima in G'/G_0 are larger than those measured for hydrogels filled with bare core/shell nanoparticles. All gels showed the Payne effect, as can be seen in the inset to figure 4.46, for the hydrogel and magnetogels doped with 30 m/m% nanoparticles. The Payne effect has been reported for a large variety of viscoelastic matrices reinforced by solid fillers and is characterized by a local maximum in G'' , which is concomitant with a significant decrease of G' with the increasing strain. Such effect can be associated with the breakage and recovery of weak interaction bonds linking adjacent filler clusters, the aggregation-disaggregation of nanoparticles or the molecular disentanglement [59]. Nevertheless, data displayed in figure 4.46 indicate that the nonlinear viscoelastic response of samples is sensitive to differences in particle-particle and particle-hydrogelator interactions.

4.5. References

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Chapter 5 – Conclusion and future perspectives

5.1. Conclusion

In this work, core/shell manganese ferrite/gold and gold-decorated manganese ferrite nanoparticles were developed through direct growth of a gold shell and covalent linkage of gold nanoparticles to manganese ferrite nanoparticles, respectively.

Considering the sulphur and amine affinity towards gold, two hydrogelators comprising either a thioether or amine moiety were synthesized and physicochemically characterized. Here, a polarity gradient of the hydrogelator structure was observed to favour gelation while the zwitterionic character of the lysine containing hydrogelator led to gelation conditions out of the scope of biomedical interest. The hydrogelators were computationally evaluated as potential anti-inflammatory agents and molecular properties were demonstrated to be dominated by the naproxen moiety.

The magnetogels were successfully obtained for a limit of 10 m/m% of nanoparticles, above which sedimentation was faster than gelation. The nanoparticles induced an increase of random coil and decrease of ordered structures, and no major effect over the chiral stacking of naproxen moieties.

The systems showed to be suitable for encapsulation of curcumin, though gels of Npx-L-Lys-Z Δ Phe-OH led to curcumin degradation owing to the basic pH conditions required for gelation, which is inadequate for drug delivery. The presence of nanoparticles improved the encapsulation as it induced more apolar regions in the hydrogel matrix. Moreover, interaction of the gels with biomembrane models showed that curcumin can be delivered but no penetration of vesicles into the gel matrix occur, owing to the high density of fibres and small aqueous cavity size.

Gold-decorated manganese ferrite nanoparticles were more efficient in the generation of heat compared to the core/shell manganese ferrite/gold nanoparticles, according to the obtained results using curcumin as a fluorescence thermometer. However, drug release by irradiation was only enhanced in the core/shell nanoparticle-containing magnetogels, though delivery was higher in both irradiated gels compared to non-irradiated gels.

The superparamagnetic properties of the nanoparticles are adequate for biomedical application and the gels elastic properties fall in the range of soft tissues. Therefore, the magnetogels based on Npx-L-Met-Z Δ Phe-OH are promising for multimodal cancer therapy by combining controlled drug delivery and photothermia.

5.2. Future perspectives

Considering the interest of Npx-L-Lys-Z Δ Phe-OH amine moiety that can bind gold surfaces, it will be evaluated if the combination with other hydrogelators allows the preparation of supramolecular plasmonic magnetogels at neutral pH.

Gold-decorated nanoparticles showed promising results for photothermia, though the plasmon band is around 600 nm, which cannot be efficiently excited in deeper tissues. Thus, gold-decorated nanoparticles will be synthesized with nanorods to shift the plasmon band to higher wavelengths. However, functionalization should be carried out with longer ligands to improve nanoparticles stability so that a higher content of nanoparticles can be encapsulated in the hydrogels matrix.

The high elasticity of Npx-L-Met-Z Δ Phe-OH at 0.3 wt% suggest that the number of aromatic moieties can be decreased and still obtain a hydrogel. Hereby, the compounds Z-L-Met-Z Δ Phe-OH, Z-L-Met-Z Δ Abu-OH and Z-L-Met-Z Δ Ala-OH will be synthesized, and gelation will be assessed.

Manganese ferrite nanoparticles have great superparamagnetic properties, but the presence of heavy atoms is undesirable due to the associated toxicity, thus it is required the development of biocompatible nanoparticles such as MgFe₂O₄ or CaFe₂O₄, stable and with similar magnetization without requiring calcination that is of interest for large scale production.

Both photothermia and magnetic hyperthermia can be used to induce drug release. Therefore, it will be evaluated which is more efficient in heat generation and triggered drug release, and if the combination of both properties in magnetic/plasmonic nanoparticles can enhance each effect.

The magnetogels will be evaluated as MRI contrast agents at various concentrations of nanoparticles and hydrogelator to assess the effect of network density on the contrast. The presence of a gold shell further allows the use of photodynamic therapy that can be enhanced in combination with photothermia.

Considering the biological applicability of magnetogels, biological assays will be carried out to evaluate the controlled drug release influence on tumour cell death with and without photothermia or magnetic hyperthermia.

Annexes

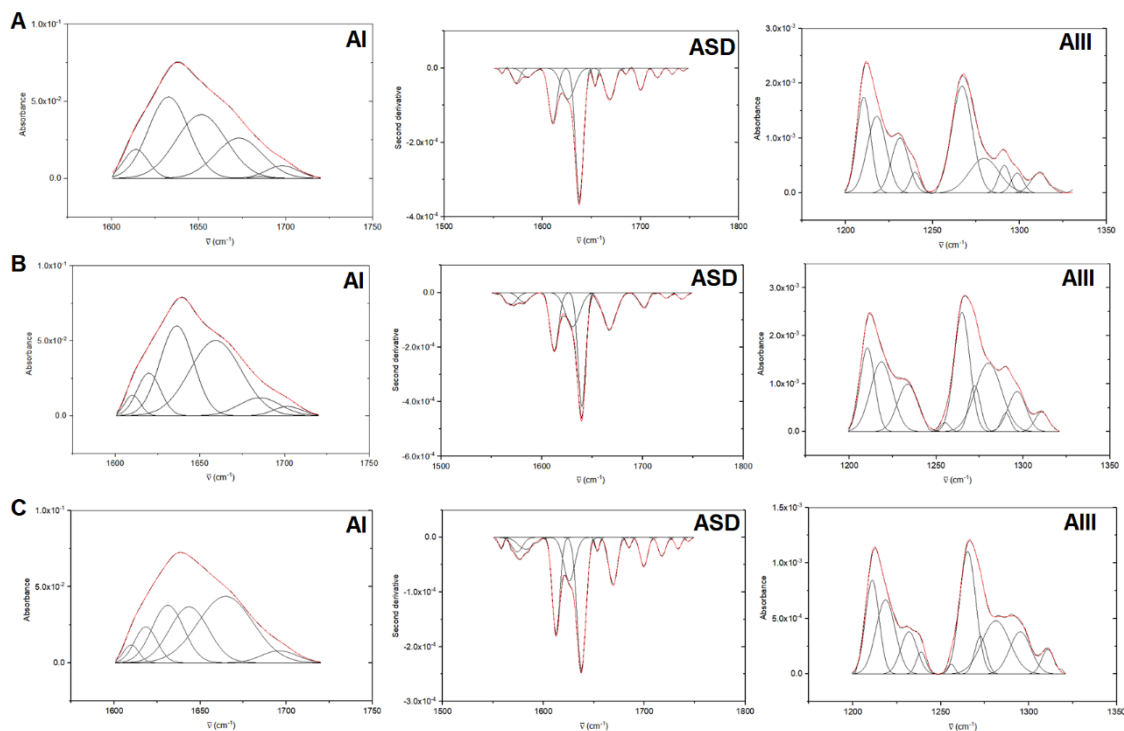


Figure A1. FTIR spectra of 1 wt% Npx-L-Met-Z Δ Phe-OH hydrogel (A), and magnetogels containing 10 m/m% core-shell (B) or decorated (C) nanoparticles. Legend: AI is amide I band; ASD is second derivative of amide I band; AIII is amide III band.

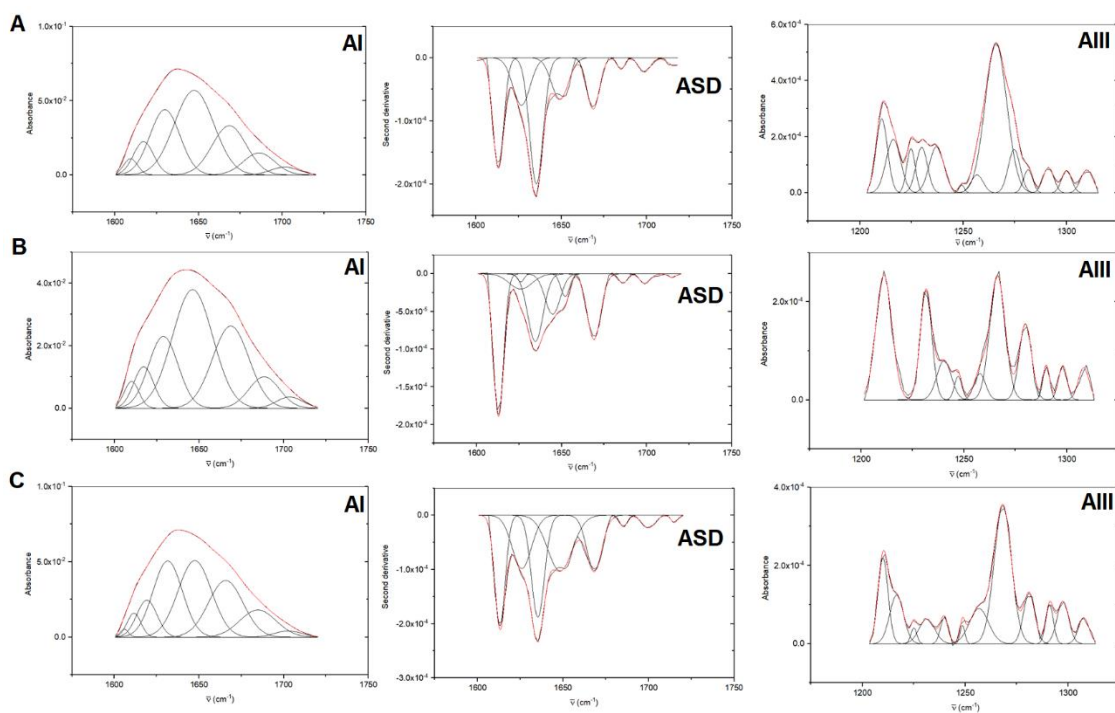


Figure A2. FTIR spectra of 1 wt% Npx-L-Lys-Z Δ Phe-OH hydrogel (A), and magnetogels containing 10 m/m% core-shell (B) or decorated (C) nanoparticles. Legend: AI is amide I band; ASD is second derivative of amide I band; AIII is amide III band.

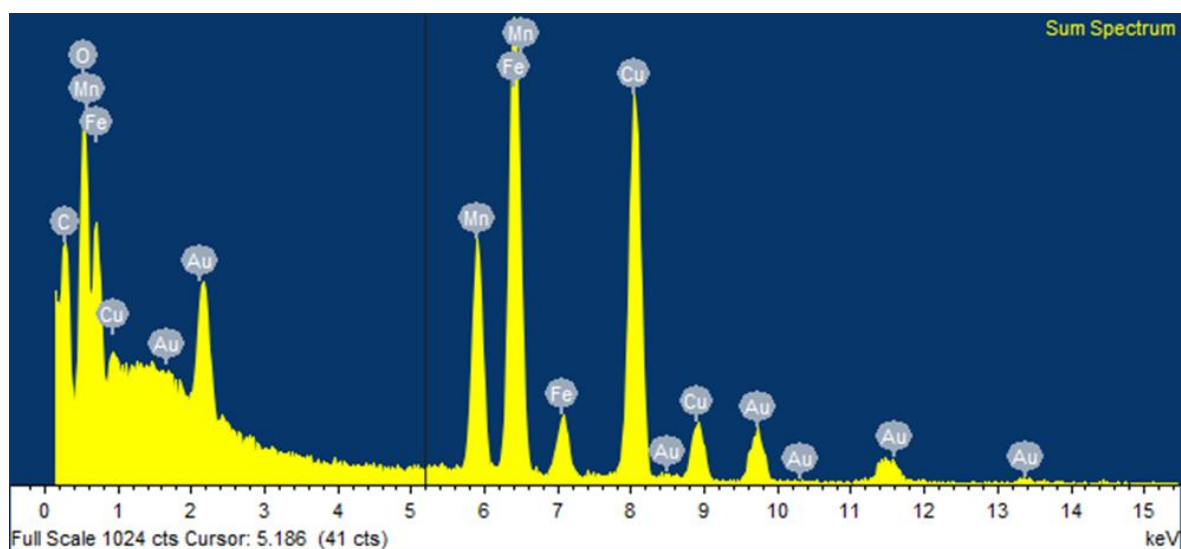


Figure A3. Electron dispersive X-ray spectrum of core/shell manganese ferrite/gold nanoparticles.

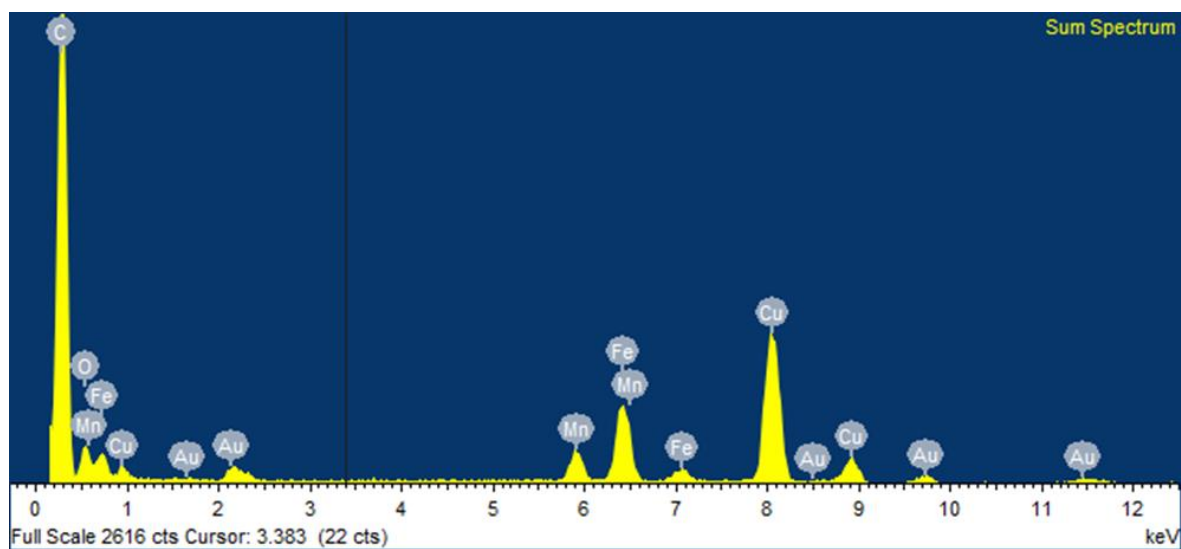


Figure A4. Electron dispersive X-ray spectrum of gold-decorated manganese ferrite nanoparticles.

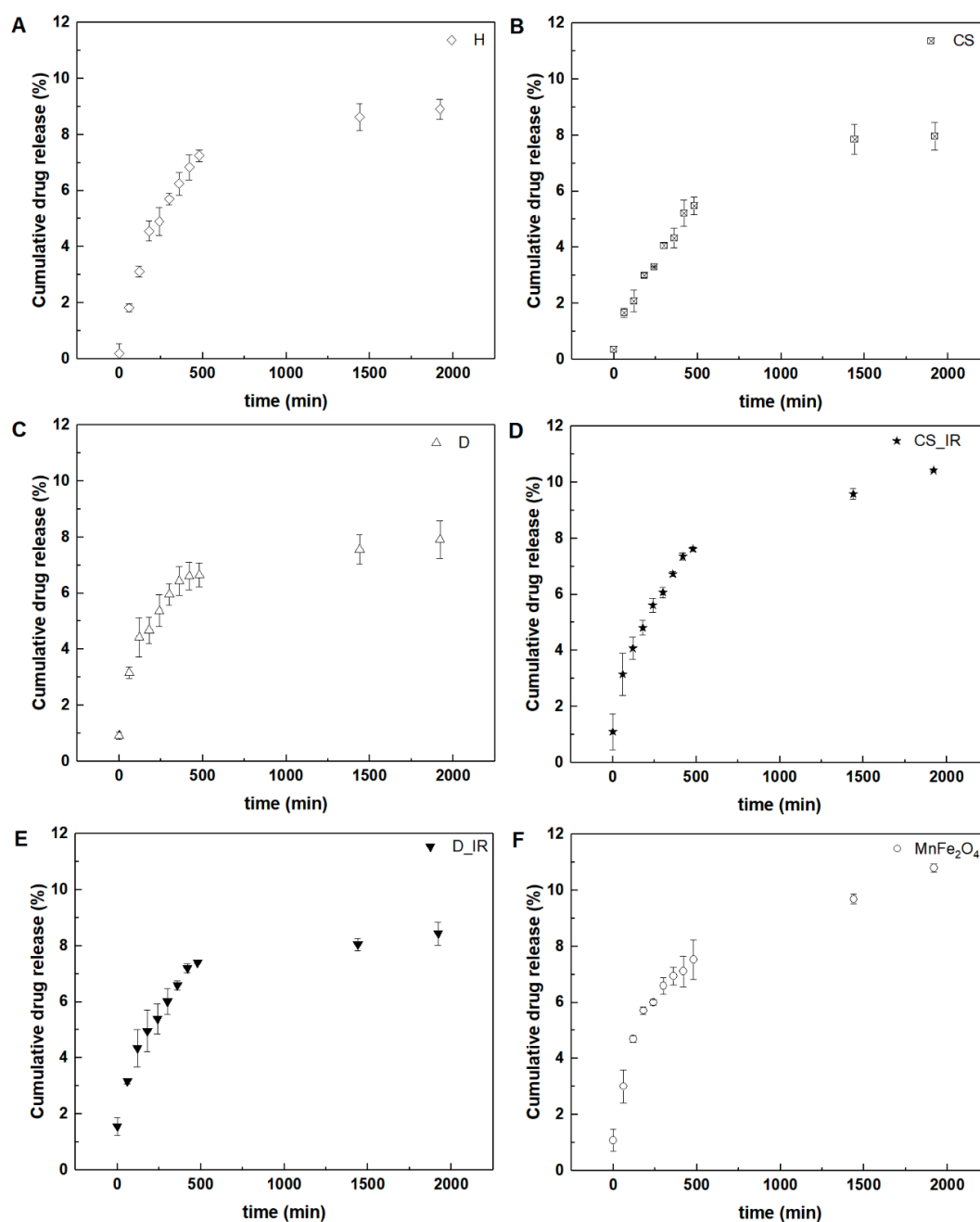


Figure A5. Cumulative drug release profiles and the respective standard deviation of curcumin (20 μ M) in 0.3 wt% Npx-L-Met-Z Δ Phe-OH hydrogel (H), and magnetogels containing 10 m/m% core-shell (CS) or decorated (D) nanoparticles with and without incidence of radiation (IR) and MnFe₂O₄ nanoparticles.

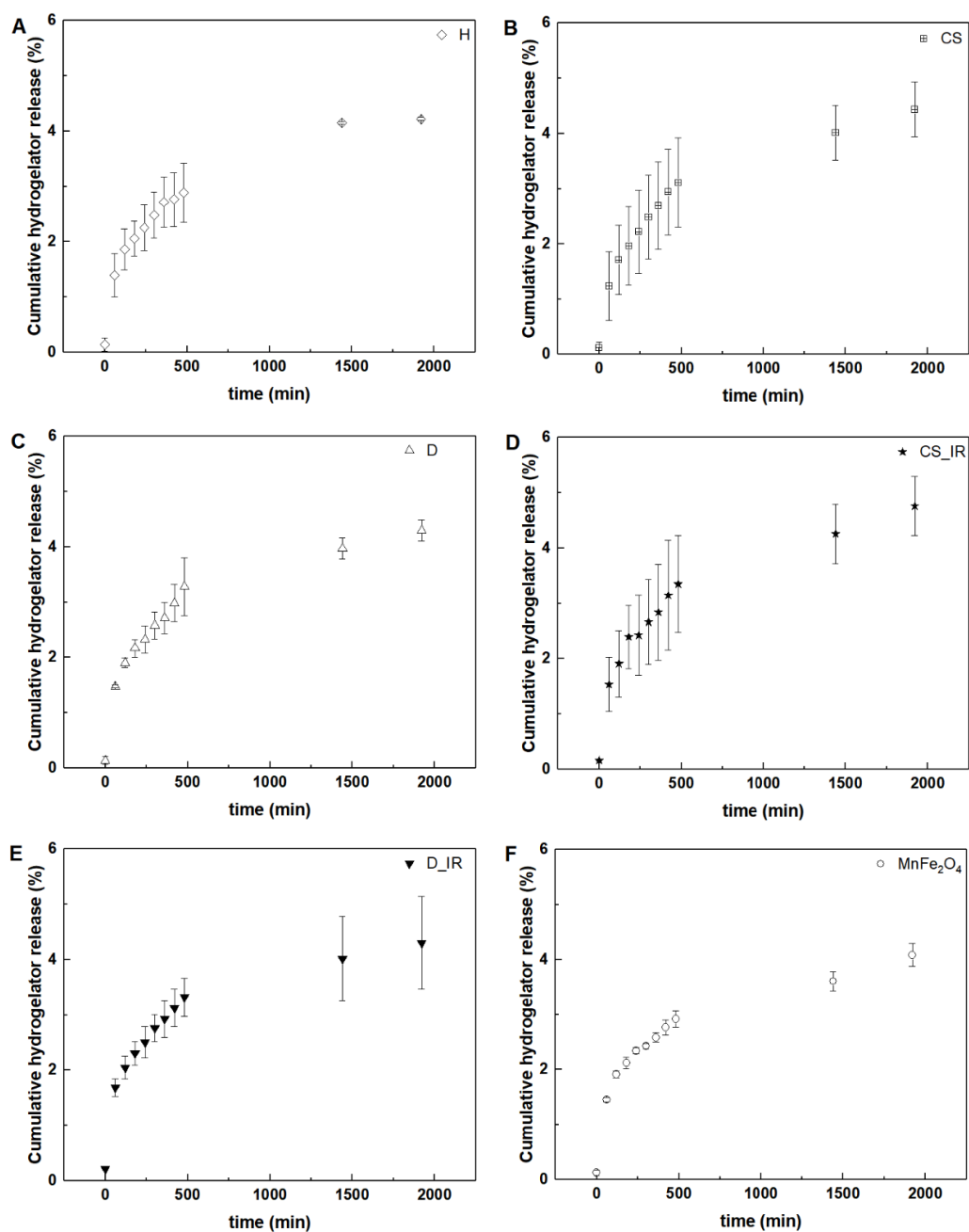


Figure A6. Cumulative hydrogelator release profiles and the respective standard deviation of 0.3 wt% Npx-L-Met-Z Δ Phe-OH hydrogel (H), and magnetogels containing 10 m/m% core-shell (CS) or decorated (D) nanoparticles with and without incidence of radiation (IR) and MnFe₂O₄ nanoparticles.

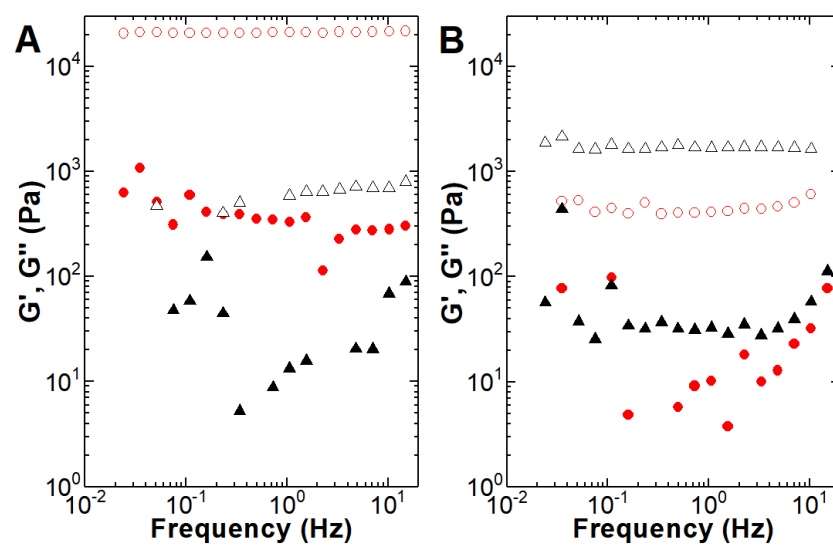


Figure A7. Mechanical spectra (G' , empty symbols; G'' , solid symbols) of the gels formulated with 10 m/m% (circles) and 20 m/m% (triangles) of (A) gold-decorated nanoparticles or (B) core/shell nanoparticles.