DEVELOPMENT OF MULTIFUNCTIONAL SUPRAMOLECULAR MAGNETOGELS FOR MULTIMODAL CANCER THERAPY

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Summary Report and Work Plan (third year) of the Doctoral Program in Physics (MAP-fis)

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Resume

In line with the work plan proposed for the first and second years of activities under the PhD grant SFRH/BD/144017/2019, it was predicted the starting of Tasks: 1 (synthesis and characterization of spherical and anisotropic magnetic nanoparticles); 2 (development of multifunctional hydrogels and magnetogels loaded with drugs) and 3 (evaluation as multimodal platforms). Hereby, a detailed description of the developed work in each task is here included, as well as a discussion of the work plan progress accomplished so far.

Task 1: Synthesis and characterization of spherical and anisotropic magnetic nanoparticles

Citrate-stabilized magnetic nanoparticles based on $X_nY_{1-n}Fe_2O_4$ (X=Ca, Mg; Y= Mn; n=0, 0.5, 1) were synthesized through co-precipitation and functionalized with amino acids. The manganese ferrites synthesized in the presence of citrate or lauric acid were characterized (structure and magnetic properties) and the results were published in the article [1] and presented in a poster communication [2]. The effect of doping with calcium or magnesium of the manganese ferrites synthesized in the presence of citrate is going to be published in the submitted article [3]. The addition of magnesium had a detrimental effect on the magnetization of citrate-stabilized nanoparticles synthesised through co-precipitation, while it remained closely unaffected or improved by doping with calcium. Despite the similar saturation magnetization, the smaller size of the synthesised calcium-doped manganese ferrites compared with the manganese ferrites by the same method led to an inferior intrinsic loss power (ILP). Hereby, further synthesis of $X_nY_{1-n}Fe_2O_4$ (X=Ca, Y= Mn; n=0, 0.5, 1) is being carried out through co-precipitation using citrate and other ligands (e.g. poly(ethylene glycol)) as stabilizing agents to obtain small nanoparticles (~20 nm) with high saturation magnetization and heating efficiency. Besides, different thermal decomposition methods are being used to either obtain spherical or cubic nanoparticles with easy tuneability and high crystallinity, so to further improve the magnetic properties. The commonly employed techniques for characterization have been the X-ray diffraction (XRD), transmission electron microscopy (TEM), superconducting quantum interference device (SQUID) magnetometer, and magnetic hyperthermia efficiency (determined through the calorimetric method). A review of the state-of-art of the fabrication strategies and applications of anisotropic magnetic nanoparticles was published in [4]. A book chapter on magnetic and plasmonic nanoparticles advances in theranostic applications was published in [5].

Task 2. Development of drug-loaded multifunctional hydrogels and magnetogels

Task 2.1. Synthesis and characterization of novel hydrogels

The development of carboxybenzyl-protected dehydropeptides with variable chemical composition led to the conclusion that the peptide sequence phenyldehydrophenylalanine enables attaining gel with thermal reversibility and low critical gelation concentration. The gelators based on the carboxybenzyl protection group were characterized and published in the article [6]. Hereby, a hydrogelator based on dehydropeptide *N*-protected with 2-naphthylacetyl was synthesised to further improve the properties by increasing the number of available aromatic groups. The new hydrogel was thoroughly characterized and is going to be published in the submitted article [3]. Having in mind the need to improve the biological properties, the synthesis of RGD (arginylglycylaspartic acid) epitope was achieved through solid phase synthesis and was coupled to the synthesised hydrogelator *N*-protected with 2-naphthylacetyl. Besides the abovementioned advancements, a dynamic simulation contribution was carried out for article [7], and the results of the developed carboxybenzyl-protected dehydropeptide-based hydrogels were presented in [8,9].

Task 2.2. Development and characterization of magnetogels

Magnetic gels based on the carboxybenzyl-protected hydrogelators containing citrate or lauric acid-stabilized nanoparticles were characterized and published in [1] and presented in poster communication [2].

Magnetic gels based on the 2-naphthylacetyl-protected dehydropeptide containing amino acid-stabilized magnetic nanoparticles were developed and characterized. The characterized magnetogels will be published in the article [3]. The use of nanoparticles functionalized with aromatic groups and amine moieties ((di)phenylalanine) enabled the co-assembly with the hydrogel fibres, which strongly enhanced the gelation kinetics. This was in contrast with the previously used negatively charged nanoparticles (stabilized with citrate), but in close similarity with the use of lipid coated nanoparticles, which also displayed nanoparticles distributed along the hydrogel fibres. Therefore, was concluded that the nanoparticles need to strongly interact with the hydrogel fibres through aromatic and electrostatic interactions to favour the co-assembly.

Task 2.3. Development and characterization of magnetolipogels

To assess the role of the combination of magnetic nanoparticles and liposomes, a review of literature on the advancements and biomedical applications of magnetoliposomes was carried out [10].

As an initial proof-of-concept, solid and aqueous magnetic liposomes were combined with a library of dehydropeptide-based hydrogels protected in the *N*-terminal with naproxen, which results were published in [11] and communicated in an oral presentation [12]. The combination of solid and aqueous magnetoliposomes in gels demonstrated that there is a partition of hydrophobic drugs between the lipid

membrane and gel's fibres. Further, the use of magnetoliposomes seemed to be counterproductive as solid magnetoliposomes can only carry hydrophobic drugs, and the aqueous magnetoliposomes display a limited loading of hydrophilic drugs due to occupation of the aqueous inner lumen by the magnetic nanoparticles. Hereby, the main strategy to optimize the encapsulation of both hydrophilic and hydrophobic drugs was to explore the close proximity or association of nanoparticles with the external layer of liposomes, thus leaving the drug encapsulation by the liposomes unaffected. Such was achieved by making use of 2-naphthylacetyl-protected dehydropeptide hydrogel, which results were included in the article [3]. Besides, in this work, the use of PEGylated liposomes was observed to strongly enhance the gelation kinetics. The results from the combination of solid and aqueous magnetoliposomes in gels, and some of the recent results in combination of liposomes with supramolecular magnetic gels were presented in oral presentation [13]. In addition, a review of the state-of-art on the advancements in the development and application of magnetogels and magnetolipogels was published [14], which resulted from the work for the Curricular Unit Essay of the MAP-fis doctoral program.

The possibility of using gold nanoparticles (spheres or nanorods) associated with liposomes and posterior combination with hydrogels was also evaluated as a means to improve the control over drug release. In this work, through the use of gold nanospheres, it was possible to study different preparation methods, which revealed the importance of initially combining liposomes with nanoparticles and then the hydrogelator solution. The active control over a hydrophilic model drug through photothermia was only achieved by its encapsulation in liposomes. The results are going to be published in the submitted article [15].

Task 3. Evaluation as multimodal platforms

The commercial chemotherapeutic drug doxorubicin was encapsulated in the gels published in references [1], [3] and [6], and the release in physiological pH was studied with and without a temperature and low frequency alternating magnetic field.

Curcumin has also been used as a model drug, which release from solid and aqueous magnetoliposomes encapsulated in gels was hampered compared to the curcumin encapsulated only in gels. Contrarily, magnetolipogels loaded with doxorubicin in the liposomes (and free doxorubicin in the hydrogel matrix) displayed higher triggered release than the magnetogels owing to the encapsulation of doxorubicin reducing the concentration available to participate as a cross-linker. Besides the controlled drug release, the magnetic hyperthermia efficiency of the nanoparticles embedded in the magnetic gels was also evaluated and reported in articles [1] and [3], while the photothermia capability was reported in article [15].

Regarding the gels components cytocompatibility, i.e. the hydrogelator and magnetic nanoparticles, were demonstrated to be cytocompatible in references [3] and [6]. Particularly, calcium-doping of manganese

ferrites was observed to further reduce the nanoparticles' potential toxicity in the 12.5-100 μg/mL concentration range, which was further improved by functionalization with (di)phenylalanine.
In addition, two oral communications were carried out on plasmonic magnetogels as multimodal platforms [16,17] and another two on the advances with supramolecular magnetic gels [18,19].

Other activities

Besides the abovementioned tasks, the student provided a workshop on the fabrication and study of supramolecular hydrogels [20], lectured a 4 hours class on Raman spectroscopy included in the Curricular Unit "Técnicas Avançadas em Biofísica II" (Advanced Techniques in Biophysics II) of the Master in Biophysics and Bionanosystems of the University of Minho [21], and participated in an e-workshop on "Docking, QSAR and Molecular Dynamics Simulations" [22], and on "Molecular Dynamics Simulations for Beginners" [23]. An article on plasmonic magnetic gels was finalized and published in [24], which resulted from previous work. In addition, the student reviewed an article for the journal *Nanomaterials* and another for the journal *Bioengineering* of the publisher *MDPI*.
Hereby, it is concluded that the objectives proposed for the first and second year of the work plan under the grant SFRH/BD/144017/2019 were accomplished.

Summary of the advancements

Initially, through an expedite and scalable synthesis strategy, a library of Cbz-protected dehydrodipeptides Cbz-*L*-Xaa-*Z*-ΔPhe-OH (Xaa= Met, Phe, Tyr, Ala, Gly) was synthesised and evaluated as minimalist hydrogels [6]. The Cbz-*L*-Tyr-*Z*-ΔPhe-OH, Cbz-*L*-Met-*Z*-ΔPhe-OH and Cbz-*L*-Phe-*Z*-ΔPhe-OH compounds successfully formed hydrogels (although only the two latter have mechanical properties of biological interest), pointing out for the relevance of a bulky hydrophobic group close to the aromatic *N*-capping group. Particularly, Cbz-*L*-Phe-*Z*-ΔPhe-OH could form thermoresponsive gels at physiological pH. In general, the gels displayed similar preparation conditions, secondary structure convergence, critical concentration of self-assembly and gelation associated with the similar chemical structure, but different kinetically-trapped state morphologies.
Regarding the use as drug carriers, the drug release profiles and FRET studies of drug transport into small unilamellar vesicles (as biomembrane models) demonstrated that the hydrogels were effective nanocarriers for drug delivery of the hydrophobic model drug curcumin and the chemotherapeutic drug doxorubicin. Mainly, the Cbz-*L*-Phe-*Z*-ΔPhe-OH hydrogel displayed an improved controlled drug release of doxorubicin, while a similar curcumin release profile was obtained compared to Cbz-*L*-Met-*Z*-ΔPhe-OH hydrogel.
Following the new library of gelators, two different functionalized nanoparticles, citrate-stabilized and lipid-coated magnetic nanoparticles, were used for the formation of dehydropeptide-based supramolecular

magnetogels consisting of the ultra-short hydrogelator Cbz-*L*-Met-*Z*ΔPhe-OH to assess their effect over gel properties [1]. The lipid-coated nanoparticles were distributed along the hydrogel fibers, while citrate-stabilized nanoparticles were aggregated upon gelation. The different nanoparticle distribution impacted differently the resulting heating and controlled drug release properties. For instance, a heating efficiency improvement and decrease was obtained for the lipid-coated and citrate-stabilized nanoparticles, respectively, besides that the former did not affect drug encapsulation and displayed improved drug release reproducibility compared to citrate-stabilized nanoparticles. In this sense, the adsorption of nanoparticles to hydrogel fibers can be explored as a means to optimize the supramolecular magnetogels for drug delivery applications.

Regarding the integration of liposomes as storage units in gels, two magnetoliposome architectures, solid and aqueous, were combined with a library of supramolecular peptide-based hydrogels [11]. This proof-of-concept was carried out through combination of magnetoliposomes (loaded with the model drug curcumin and the lipid probe Nile Red) with the hydrogels prior to pH triggered gelation. The encapsulated molecules distributed to similar environments independently of the magnetoliposome architecture. Besides, the extra hydrophobic cavities provided by the liposomes enabled a slower release of curcumin compared to the neat hydrogels.

From the abovementioned observations, a novel dehydropeptide hydrogelator was synthesized N-capped with 2-naphtaleneacetic acid, 2-Naph-L-Phe-*Z*ΔPhe-OH, to improve the thermal stability and achieve a lower critical gelation concentration through the increase of aromatic moieties [3]. A new design strategy was explored to modulate the release of doxorubicin (known to co-assemble as spherical cross-units with peptide-based hydrogels) through the interplay of (di)phenylalanine-coated (providing hydrophobic, electrostatic and aromatic interactions) magnetic nanoparticles, PEGylated liposomes and doxorubicin co-assembly in dehydropeptide-based gels. The integration of liposomes as doxorubicin storage units and of nanoparticles as composites that co-assemble with the gel matrix enabled the tuneability of both passive and active doxorubicin release through a thermal, or a low-frequency alternating magnetic field-based trigger. In addition to the modulation of the gel properties, the functionalization with (di)phenylalanine improved the cytocompatibility of the nanoparticles.

Later, seeking a different stimulus so to achieve multimodal sequential release, the fabrication of particle-liposomes assemblies was explored as a means to achieve stimuli-responsive storage units [15]. In this work, the active control over a hydrophilic model drug, 5(6)-carboxyfluorescein, was only achieved by its encapsulation in liposomes, in which the presence of silica-coated gold nanorods further enabled the use of hyperthermia with near-infrared laser irradiation. Besides, in this work, the release of liposomes was also evidenced as a pathway to deliver the loaded cargo, and the use of gold nanoparticles enabled the study of the preparation method, which revealed the importance of initially combine liposomes with nanoparticles and then the gelator solution, to ensure the proximity of the nanoparticles to the liposomes.

Work plan foreseen for the third year of the PhD doctoral program

Considering the previously described undertaken advances, the next work has as major objectives: (1) achieve an injectable gel through exploration of the preparation methods of the novel hydrogelator, 2-Naph-L-Phe-*Z*-ΔPhe-OH; (2) endow the gel fibres with targeting properties through incorporation of gelator molecules with RGD epitope; (3) achieve the individual compartmentalization of three antitumor drugs (paclitaxel, doxorubicin and methotrexate); (4) induce the sequential release of each loaded drug. Therefore, the following work plan is proposed for the third year of the doctoral program according to the previously proposed tasks.

Task 1: Synthesis and characterization of spherical and anisotropic magnetic nanoparticles

Further synthesis of magnetic nanoparticles based on $X_nY_{1-n}Fe_2O_4$ (X=Ca, Y= Mn; n=0, 0.5, 1) is being carried out through co-precipitation using a mixture of the metal ions salts, citrate and other ligands (e.g. poly(ethylene glycol)) as stabilizing agents to obtain small nanoparticles (~20 nm) with high saturation magnetization and heating efficiency. So far, the calcium-doped manganese ferrites have displayed promising magnetic and biological properties, thus being of interest for further functionalization and co-assembly with the supramolecular hydrogels. However, considering the need to further improve the heating efficiency, different thermal decomposition methods are being used to either obtain spherical or cubic nanoparticles with easy tuneability and high crystallinity. The magnetic nanoparticles structural properties will be studied mainly through HR-TEM, S(T)EM and XRD, while magnetic properties will be assessed either through superconducting quantum interference device (SQUID) or a vibrating-sample magnetometer (VSM).

Task 2. Development of drug-loaded multifunctional hydrogels and magnetogels

Task 2.1. Synthesis and characterization of novel hydrogels

In line with the work plan proposed for the doctoral program and the described advancements, supramolecular peptide-based compounds *N*-capped with an aromatic group 2-naphthylacetyl and a dehydroamino acid in the *C*-terminal (dehydrophenylalanine), will be synthesized. Further, the synthesis of a RGD epitope will be carried out through solid phase peptide synthesis using a 2-chlorotrityl chloride resin, which will be added *N*-capped dehydrodipeptide synthesised in [3]. The hydrogels will comprise a mixture of the dehydrodipeptide with/without the RGD epitope, enabling the modulation of the final gel's properties by varying the composition of the co-assembly mixture. To assess the possibility of achieving injectable gels, different preparation methods are going to be studied, including the heating-cooling and solvent exchange methods.

Task 2.2. Development and characterization of magnetogels

Magnetic gels based on the 2-naphthylacetyl-protected dehydropeptide with/without the RGD epitope containing amino acid-stabilized magnetic nanoparticles will be developed and characterized. The use of nanoparticles functionalized with aromatic groups and amine moieties ((di)phenylalanine) was demonstrated to enable the co-assembly with the hydrogel fibres, and enhance the gelation kinetics, thus are intended to be included as composites in the subsequent systems. The gels mechanical properties will be studied through rheology assays, and the physicochemical properties will be mainly focused on the use of fluorescence to assess changes in the π-π stacking, microviscosity and gel-sol transition temperature (assessed using fluorescence anisotropy). Other techniques required for physicochemical characterization include Raman spectroscopy, ATR-FTIR and circular dichroism. Molecular dynamics will also be used to study the potential interaction between the dehydrodipeptides with/without RGD epitope, simulate the interaction of the dehydropeptides with biological membranes and integrins (the receptor protein of the RGD epitope). The heating efficiency of the magnetic nanoparticles will be studied through the calorimetric method.

Task 2.3. Development and characterization of magnetolipogels

The main strategy to fabricate magnetolipogels will consist on the gelation of hydrogels in the presence of aqueous magnetoliposomes, with and without non-encapsulated nanoparticles available to participate in the co-assembly with the gel network. The liposomes will contain a mixture of phospholipids labelled with the RGD epitope (and/or PEG) to enhance its interaction with cancer cells, and thus improve the delivery of the loaded antitumor drugs. The assessment of the liposomes stability will include the use of dynamic light scattering (DLS) to determine the hydrodynamic diameter, polydispersity and zeta potential, as well as fluorescence spectroscopy (using suitable fluorescence probes) to study changes in the membrane dynamics upon gelation and interaction with the dehydropeptides, drugs and nanoparticles. In addition, the magnetolipogels will also incorporate gold nanorods coated with mesoporous silica in the gel matrix. These composites will be loaded with different drugs (paclitaxel, doxorubicin and methotrexate) as a means to achieve the sequential release of the loaded cargo using different stimulus.

Task 3. Evaluation as multimodal platforms

The optimized injectable magneto(lipo)gels will be loaded with the previously established chemotherapeutic drugs, such as doxorubicin (breast, stomach, bladder and thyroid cancers, lymphoma, myeloma), paclitaxel (ovarian, breast, lung, cervical and pancreatic cancer) and methotrexate (breast, advanced head and neck, lung, and stomach cancer). Through the loading of drugs in different compartments/storage units (magnetoliposomes, and gold nanorods with mesoporous silica), the

magnetogels will enable the encapsulation of both the hydrophobic and hydrophilic drugs, and ensure an on-demand drug release. The gels will be evaluated as nanocarriers by studying the localization of the drugs in the gel matrix (fluorescence emission and anisotropy) and the interaction with biomembrane models will be followed through the Förster resonance energy transfer process (FRET) using appropriate fluorescent probes. The heating profiles (and efficiency) upon photothermia and magnetic hyperthermia, will be evaluated through the calorimetric method, i.e. the sample is subjected to a stimulus (a high-frequency alternating magnetic field or laser irradiation) and temperature is measured by a probe (usually a fibre-optical thermometer). Once the sequential and on-demand drug release profiles are well described, and the heating efficiencies achieved through photothermia and magnetic hyperthermia are optimized, the systems' drug delivery efficiency and efficacy are going to be assessed in *in vitro* assays in cancer cell lines.

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