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Preface

MEDICON 2019 is the XV in the series of regional meetings of the International Federation of Medical and Biological Engineering (IFMBE) in the Mediterranean. MEDICON 2019 will be organized for the first time in Portugal and will be hosted by the UNESCO World Heritage University, the University of Coimbra.

The goal of MEDICON 2019 is to provide updated information on the state of the art on Medical and Biological Engineering and Computing under the main theme "**Patient empowerment**". Patient empowerment has emerged as a new paradigm that positions the patients at the heart of the health system and encourages them to be actively involved in managing their own healthcare needs. Effective patient empowerment requires a holistic approach, combining multiple dimensions of needs and patient contexts. Medical and Biological Engineering and Computing is a discipline at the heart of patient empowerment. Research and development in these areas are impacting the science and technology by advancing fundamental concepts in translational medicine and understanding in human physiology, function and behaviour at multiple levels. This is leading to improved tools and techniques for the detection, prevention, treatment and management of diseases. MEDICON 2019 provides a common platform for the cross fertilization of ideas and to help shape knowledge and scientific achievements by bridging complementary disciplines into an interactive and attractive forum under the special theme of the conference that is "improving health care through holistic patient empowerment".

The programme consists of some approximately 250 invited and submitted papers on new developments around the Conference theme, presented in 8 plenary sessions, 18 parallel scientific sessions and 19 special sessions and also includes a set of competitions and awards.

More specifically, the parallel scientific sessions cover the topics of biomedical signal processing; biomedical imaging and image processing; bio-instrumentation, bio-senso and bio-micro/nanotechnologies; bioinformatics, computational biology and systems biology; biomechanics, robotics and rehabilitation; therapeutic and diagnostic systems, devices and technologies and clinical engineering; information technology in health systems; assistive technologies; technologies for active ageing;

biomedical engineering education and society; clinical engineering and health technology assessment; neuroengineering, neurosystems; technologies for preventive health care; biomedical technologies for developing countries; standardization of open data; biomaterials and tissue engineering.

The special sessions include the topics of optimization in medicine and biology; ontological engineering in biomedical informatics; electronics and smart algorithms for the effective lung monitoring and COPD management; non-invasive temperature assessment using ultrasound; computational biology and medical applications; smartphone-based, patient-centred technologies; computational and experimental modelling for designing bone-implant systems; artificial organs: extracorporeal blood circulation medical devices; diabetes and cardiovascular diseases: Ibero-American trends; smart robotic assistant for minimally invasive surgery: the SMARTsurg project experience; intelligent computational systems in biomedical engineering; INT4DAT - Intelligent systems and technologies for diagnostic, assistance and therapeutics; upper limb exoskeletons for a better quality of life: what is real, what is useful, and what is next?; neurosystems and connectivity; therapeutic applications of imaging and neurostimulation; value-based health technology assessment; international collaborative on medical device assessment; ocular imaging; assessing human error in cognitive/intellectual demanding tasks: case study on software engineering.

The conference programme also includes three competitions and two awards: IFMBE Scientific Challenge competition; Fraunhofer Best Portuguese PhD thesis competition in Biomedical Engineering; Fraunhofer Best Portuguese MSc thesis competition in Biomedical Engineering; Best Student Paper Award; Young Investigator Competition Award.

Furthermore, the conference programme is highlighted by eight plenary sessions: Digitally empowered patients, presented by Aart van Halteren; Cardiovascular modelling and simulations—applications to some clinical studies, presented by Adélia Sequeira; Biomedical signals and images processing: towards innovative paradigms of information integration in the era of precision medicine and big data in health, presented by Sergio Cerutti; Multilingual dictionary of medical physics terms—update and relevance for clinical engineering, presented by Slavik Tabakov; Prevention Engineering: evolving challenges for biomedical and clinical engineering, presented by Luis Kun; Optical coherence tomography: a window into the mechanism of neurodegenerative disorders, presented by Rui Bernardes; Towards a value based healthcare system supported by process mining techniques, presented by Vicente Traver; In silico clinical trials: towards transforming the biomedical industry and the healthcare delivery, presented by Dimitrios Fotiadis.

Particular thanks are expressed to the kind support and effort of a number of external sponsors to which we would like to express our appreciation. Finally, a heartfelt thanks to all of you, the participants for your paper contributions, wishing you every success in your work at the conference. We hope that MEDICON 2019 will offer opportunities for professional growth and establishing new contacts with

fellow colleagues. Our intention is to do all we can to make your participation in MEDICON 2019 worthwhile and your stay in Coimbra enjoyable and memorable.

We hope you will appreciate this proceedings volume as much as we are proud of it!

September 2019

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Magnetic Carbon Nanostructures and Study of Their Transport in Microfluidic Devices for Hyperthermia

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Abstract. Cancer incidence and mortality are growing worldwide at an alarming pace, emphasizing the urgent need for new strategies to combat this disease. One of the frontiers of cancer research is currently focused on the design of multifunctional magnetic nanoparticles capable to achieve the synergistic cancer theranostics (both diagnosis and therapy). Although the potentiality that these multifunctional nanosystems represents to nanomedicine, cancer treatment and diagnostic, there are still many challenges that must be addressed in a near future before this approach became a reality. The development of efficient multifunctional magnetic nanosystems able to selectively destroy cancer cells in detriment of healthy ones, is one of the main challenges that have damped the spread of this technology into clinical applications. The limited biological and biophysical studies between the biomedical nanosystems and cells/tissues/organs is another challenge that has to be addressed. With these two main challenges in mind, the present Ph.D. work was focused in the development of: (1) *Multifunctional magnetic carbon nanostructures as multifunctional nanosystems for the treatment of cancer,* and (2) *New advanced microfluidic devices capable to give new insights over the developed nanosystems and human cells*.

1 Introduction

In spite of all the progress achieved in the last decade, the translation of multifunctional therapeutic nanosystems for clinical applications, including hyperthermia and drug delivery, has been slow, especially due to the lack of robust preclinical tissue culture

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platforms able to mimic the *in vivo* conditions found in the human body and to predict the performance of the developed nanomaterials [1]. Indeed, it should be noticed that nanomaterials designed for theranostic applications have been developed to be applied directly into the bloodstream. So, the understanding of these complex cell-nanoparticle interactions is of utmost importance, especially for the development of multifunctional nanoparticles as drug delivery nanocarriers.

Currently, toxicological screening processes are performed in *in vitro* culture systems, by seeding cells within multi-well dishes. Although their merits, this methodology is not always able to predict the function and effect of drugs *in vivo*, resulting in a large number of failed drug candidates in animal experiences and clinical trials [2]. The main shortcoming of these *in vitro* cellular models, especially for the evaluation of new nanomaterials, is the inability to mimic the complex three-dimensional (3D) *in vivo* microenvironment, wherein the cells and extracellular matrix (ECM) exist in well-organized architectures [2].

Microfluidic devices, which can be defined as the set of technologies handling and processing small fluid volumes (e.g., μL, nL and pL) through microchannel geometries, with dimension of tens to hundreds of micrometers embedded in a chip [3], have several advantages to assess the biophysical properties of cells, when compared to conventional techniques. For instance, this technology allows the use of small sample volumes, integration capability of electronic systems, biocompatibility environment for cell studies, accuracy and fast response. In addition, in the recent decades, a significant advance of microfluidic technologies has allowed the design of microchannel geometries in the sizes of small vascular capillaries, which can mimic the micro-rheological properties of the blood cells in *in vitro* microvascular environment. Furthermore, the advances in computers, optics and digital image processing techniques [4], allow the assessment of haemodynamic phenomena recorded in those *in vitro* microvascular environments. More recently, a new concept of biomicrofluidic devices, called as organ-on-a-chip platforms, has been developed to mimic complex human organ functions at the microscale level. These integrated microfluidic networks, with 3D tissue engineered models, have shown the ability to reduce the discrepancies between the preclinical and clinical trials [2]. Moreover, these 3D microfluidic platforms have several advantages over the *in vivo* animal models, such as lower costs and less time-consuming, end of animal ethical concerns, visualization of the theranostic agents in the target tissues and accuracy to predict human responses.

The present Ph.D. work aimed to join these multidisciplinary approaches, envisaging new insights related to many scientific aspects of the utilization of smart magnetic nanosystems in biomedical applications, namely mechanistic and toxicological phenomena, which are essential to boost the spread of multifunctional nanoparticles as the next generation of therapeutic bio-agents in medicine.

2 Hemocompatibility of Iron Oxide Magnetic Nanoparticles for Theranostic Application: A High Sensitivity Microfluidic Tool

2.1 Development of Superparamagnetic Nanoparticles for Theranostic Applications

The development of superparamagnetic nanoparticles for nanomedicine has attracted much attention in the last decades, especially due to their remarkable physicochemical properties acquired at the nanoscale. In particular, superparamagnetic iron oxide nanoparticles have gained increasing importance in the field due to the possibility to combine diagnosis and therapy of cancer, i.e. as contrast agents for MRI and as nanoheaters in magnetic hyperthermia (MH). MH is considered a promising therapeutic technique for the treatment of cancer since it implements the remarkable nanoscale physicochemical properties of magnetic nanoparticles (MNPs) to generate heat under an alternating magnetic field (AMF). These nanoheaters can be designed to preserve healthy cells while destroying selectively tumoral ones, inherently more sensitive to mild-temperatures changes of 5 to 7 °C above the body temperature [5, 6]. Nevertheless, the poor heating efficiency of most reported MNPs, together with the lack of biocompatibility and hemodynamic studies, have hamper the spread of multifunctional nanoparticles as the next generation of theranostic bio-agents in medicine.

Herein, MNPs where explored as theranostic nanosystems for the simultaneous detection (contrast agent in MRI) and treatment of cancer (MH).

(1) *Synthesis*

Two samples of magnetite were synthesized by alkaline co-precipitation, mixing the iron precursors in the stoichiometric ratio 1:2 of iron (II) and iron (III) salts under vigorous magnetic stirring. Then, the resultant mixture was heated until the desired temperature (30 or 55 °C). At that point, a basic NH_4OH (1 M) solution was added dropwise until pH $\sim$ 9, promoting the co-precipitation of the magnetite nanoparticles. The resulting materials were labelled as MAG30 and MAG55, depending on the heating temperature used (30 or 55 °C, respectively).

Afterwards, hydrophilic MNP ferrofluids were obtained after addition of a solution of alendronic acid (Al, 50 mM, pH 10) into the colloidal suspensions of MAG30 or MAG55, by using a ratio 4:1 (v/v). The resulting MNPs@Al (MAG30@Al and MAG55@Al) were dispersed in water, sonicated for 30 s and stored at room temperature.

(2) *Results and Discussion*

(a) *Size, crystal structure and chemical composition*

The results obtained by transmission electron microscope (TEM) analysis of MAG30 and MAG55 are shown in Fig. 1, revealing that the magnetic core of the synthesized nanoparticles is nearly spherical for both samples. The increase of the synthesis temperature lead to a growth in the average diameter of the magnetic core, from 11.1 (MAG30) to 17.7 nm (MAG55), which is in good agreement with the crystallite sizes obtained by X-ray powder diffraction (XRD) analysis (data not shown).

All the reflection peaks of the XRD patterns of MAG30 and MAG55 were indexed by considering magnetite phase. No reflection peaks were observed as impurities or secondary phases. The cubic cell lattice parameter resulted to be $a \sim 8.37$ Å, in good agreement with the values reported for nanosized magnetite [8].

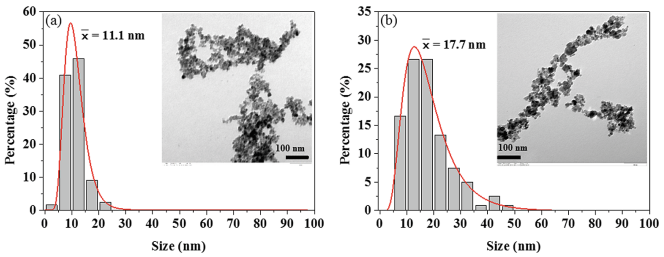

Fig. 1. Histogram and mean diameter of (a) MAG30 and (b) MAG55. Inset shows the corresponding TEM images. Reprinted from [7]. Copyright © 2016, with permission from Springer Nature.

(b) *Magnetic and hyperthermia properties*

The main magnetic parameters of MAG30 and MAG55, namely saturation magnetization, coercivity and remanence, were calculated from the analysis of the magnetization curves as a function of the applied magnetic field obtained for powdered samples (cf. Table 1 and Fig. 2).

Table 1. Magnetic properties of the nanoparticles synthesized by co-precipitation at different synthesis temperatures (30 and 55 °C): Saturation magnetization (M_s), Coercivity (H_c), Saturation remanence (M_r), Specific absorption rate (SAR) and intrinsic loss power (ILP). Reprinted from [7]. Copyright © 2016, with permission from Springer Nature

Sample	M_s (emu g^{-1})	H_c (Oe)	M_r (emu g^{-1})	SAR[a] $(W\,g^{-1}F)$	ILP (nHm^2 kg^{-1})
MAG30	56.19 ± 0.02	33.65	3.77	100.18	0.57
MAG55	77.68 ± 0.03	18.33	1.94	565.68	3.23

[a]Specific absorption rate (SAR) calculated for $H = 15.95$ kA m^{-1}, $f = 688$ kHz, C = 0.40 g $_{Fe3O4}$ L^{-1}

Both MAG30 and MAG55 hysteresis loops show a superparamagnetic-like behavior. MAG30 showed a saturation magnetization, M_s, of 56 emu g^{-1}, whereas the increase of the synthesis temperature in MAG55 led to an increase of the particle size that subsequently resulted in a higher M_s. On the other hand, the zero-field-cooling – field-cooling (ZFC–FC) magnetization curves shown in Fig. 2(b) indicate a shift of the blocking temperature (T_B), defined as the temperature above which the MNPs show superparamagnetic behavior, from –98.2 to 26.9 °C for MAG30 and MAG55, respectively. Hence, the confirmation of a superparamagnetic state for both MAG30 and MAG55 at room temperature make these samples suitable for MRI and hyperthermia applications.

(c) *Magnetic resonance relaxivity studies*

The contrast enhancement efficiency of MNPs@Al was evaluated in water through relaxivity measurements by plotting the relaxation rates ($R_1 = 1/T_1$ and $R_2 = 1/T_2$) of water protons in the presence of the nanoparticles, against the Fe content.

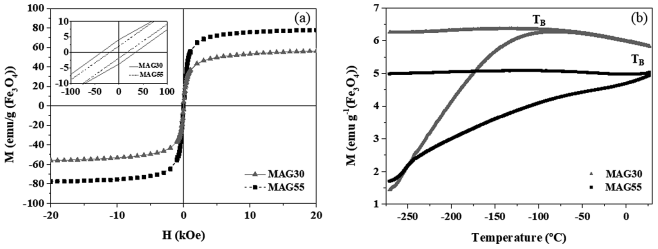

Fig. 2. Magnetic characterization of MAG30 and MAG55. (a) Hysteresis loops at room temperature up to ±20 kOe; the inset is a zoom in the low-field region; (b) ZFC-FC magnetization curves measured at 50 Oe. Reprinted from [7]. Copyright © 2016, with permission from Springer Nature.

Both MAG30@Al and MAG55@Al ferrofluidic samples showed a far superior enhanced r_2 values than currently available intravenous iron oxide nanoparticle contrast agents, i.e. 248.17 mM^{-1} s^{-1} for MAG55@Al and 226.08 mM^{-1} s^{-1} for sample MAG30@Al, indicating that both samples are promising candidates for high-efficiency T_2-weighted MR imaging.

2.2 A New Microfluidic Tool to Assess Hemocompatibility of Theranostic Nanosystems

In this study, and for the first time, a high-sensitivity microfluidic tool capable to assess the deformability of blood cells was used to evaluate the impact of nanoparticles on the human RBCs. This methodology comprises a microfluidic device having a hyperbolic-shaped contraction microchannel and a high-speed video microscopy system. This combined system enables the recording, in real time, of the biomechanical microfluidic phenomena, which can be further used to analyze valuable toxicological data.

In this type of microfluidic devices (Fig. 3), the velocity increases almost linearly and the strain rate is maintained approximately constant, even with the increment of the shear rate [9].

As a result, the technique allows the deformation of the blood cells along the microchannel, causing the RBC stretching by the larger fluid shear stress, without tumbling and rotational motions of the cells, providing a more accurate way to determine the deformation index (DI) of the blood cells, mimicking *in vitro* microvascular environments.

Figure 4 shows a healthy RBC flowing along the axial position, x, of the microchannel with a hyperbolic-shaped contraction followed by a sudden expansion, which was subdivided in four sections to assess the DI evolution of the RBCs along the microchannel.

RBCs are important for scientific and clinical purposes because they can be used as an indicative of many pathological conditions. It has been shown that changes in their normal DI are correlated to blood disorders. Thus, DIs of RBCs in contact with different concentration of MNPs at different incubation time where assessed to infer the hemocompatibility of the developed nanosystems. The DIs of the RBCs (control versus MNPs) were measured flowing along the centreline of the microchannel that was subdivided in four sections (cf. Fig. 4). The results are displayed in Fig. 5.

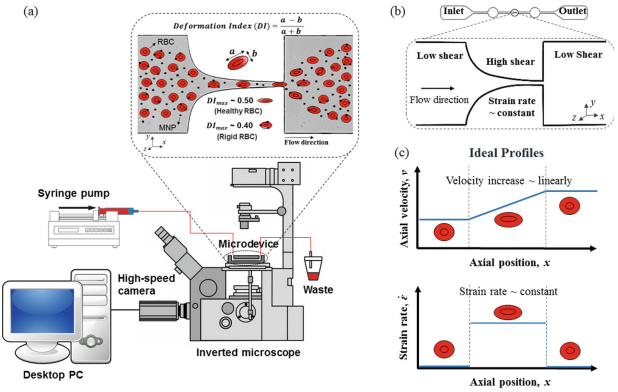

Fig. 3. Microfluidic studies for the hemocompatibility of the RBCs in contact with MNPs, (a) experimental setup; (b) microchannel device geometry with hyperbolic channel; (c) graphical representation of the fluid-induced conditions profiles that occur in the hyperbolic channel. Reprinted from [7]. Copyright © 2016, with permission from Springer Nature.

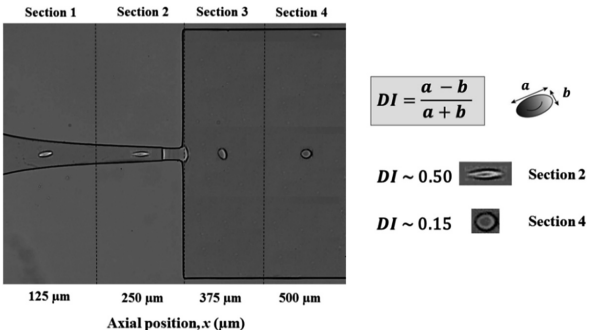

Fig. 4. Representation of a healthy RBC flowing along the axial position of a microchannel with a hyperbolic-shaped contraction followed by a sudden expansion. Reprinted from [7]. Copyright © 2016, with permission from Springer Nature.

Overall, the DI results for the RBCs samples in contact with MNPs show that the incubation time influences directly the deformability of those blood cells. Hence, the DI results allow to conclude that the increase of the incubation time between RBCs and

MNPs, lead to the decrease in DI, or in other words, to the increase of rigidity of those cells. This observation is even more obvious for the sample with the higher content of MNPs and exposure time (34.8 μg $_{Fe3O4}$ mL^{-1}, 60 min), where the DI decreased to 0.34 ± 0.02, which is a significant difference when compared with the control DI (0.45 ± 0.02).

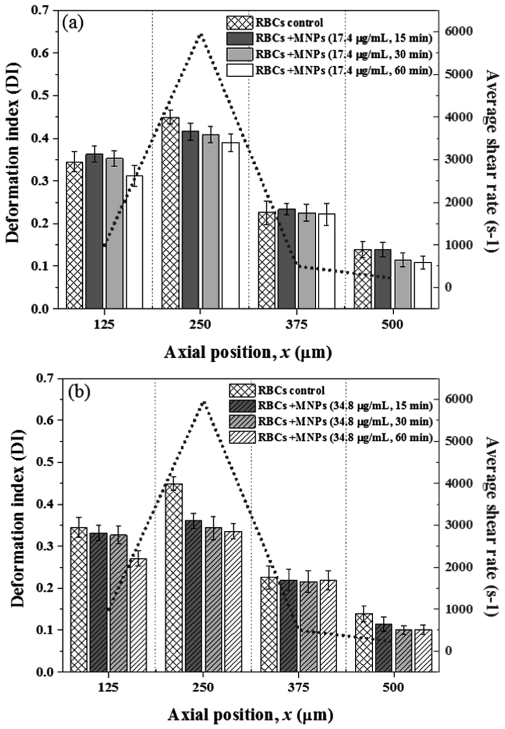

Fig. 5. DI of blood cells flowing along the axial position of the hyperbolic channel during 15, 30 and 60 min: (a) MNPs at a final concentration of 17.4 μg $_{Fe3O4}$ mL^{-1}; (b) MNPs at a final concentration of 34.8 μg $_{Fe3O4}$ mL^{-1}. Error bars show a 95% confidence interval (n = 56 blood cells). Reprinted from [7]. Copyright © 2016, with permission from Springer Nature.

This work was the first experimental test reported in the literature that corroborate the numerical work performed by Curtis et al. [10], which refers to the uptake of the surrounding MNPs by the RBC membranes, as the main reason for the increasing rigidity observed in the RBCs. In this recently numerical study, it was also predicted a dependency on the MNP size, shape and composition with the RBC membrane interaction.

This study was published in the *Journal of Nanoparticle Research* [7].

3 Graphene-Based Magnetic Nanoparticles for the Treatment of Cancer

The frontiers of cancer research are currently focused on the design of multifunctional magnetic nanoparticles capable to achieve the synergistic cancer treatment, by combining the heat effect induced by hyperthermia and their unique drug delivery properties [11]. For this purpose, many magnetic nanoparticle-based drug delivery systems have been developed in the last decade [12]. However, the major bottleneck for their combined clinical achievement has been the low drug loading capacity and poor controlled-drug release of the developed magnetic nanoparticles [13], as well as the low heating efficiency of the composite nanosystems in MH.

Herein, the synthesis and optimization of graphene-based yolk-shell magnetic nanoparticles for nanomedicine are described, as well as its efficiency as multifunctional nanosystems.

3.1 Synthesis

The synthesis procedure of graphene-based yolk-shell magnetic nanoparticles (GYSMNPs) was divided in two main steps: (i) *synthesis of the superparamagnetic core*, and (ii) *formation of the graphene-based yolk-shell architecture,* as shown in Fig. 6.

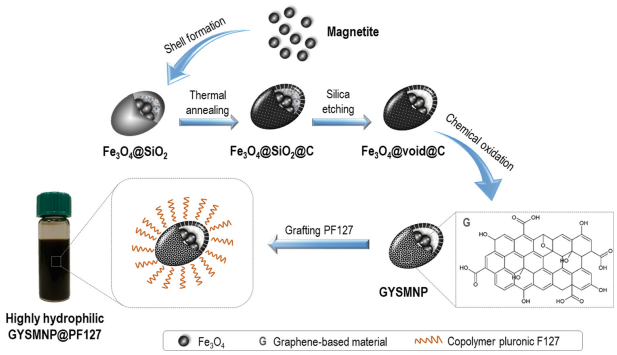

Fig. 6. Schematic overview of the steps involved in the development and grafting of the hydrophilic graphene-based yolk-shell magnetic nanoparticles (GYSMNP@PF127).

In the first step, the superparamagnetic magnetite (Fe_3O_4) core, with mean diameter of 18 nm, was synthesized through the procedure detailed in Sect. 2A, at 55 °C. Afterwards, in the second step, the graphene-based yolk-shell architecture was achieved via one-pot strategy of hydrolysis and polymerization of the precursors resorcinol, formaldehyde and TEOS. Sample was labelled as GYSMNP@PF127 after functionalization with pluronic F127 (PF127).

3.2 Results and Discussion

It is important to note that for nanomedicine, the proposed nanosystem has to show excellent colloidal stabilization in the presence of electrolyte solutions, optimal average hydrodynamic dimension (D_H), low hemotoxicity and good biocompatibility. In this context, the GYSMNP@PF127 nanosystem was further characterized taking into account those properties. D_H of GYSMNP@PF127 was found to be 180 nm, with polydispersion index (PDI) of 0.14 (data not shown). Due to the EPR effect, nanoparticles with size range of 10–200 nm are ascribed to be preferentially accumulated into tumor rather than in healthy tissues [14].

D_H of GYSMNP@PF127 shows an optimal dimension for suitable intravenous administration and EPR effect with prolonged blood circulation.

Figure 7 shows the Fourier transform infrared (FTIR) spectra of the samples collected at different stages of the preparation process.

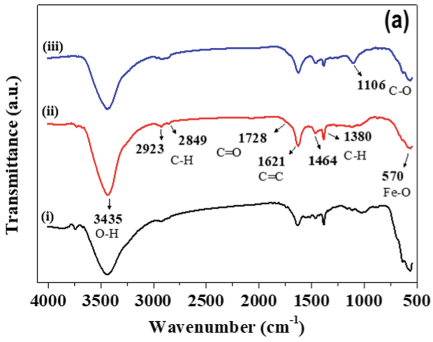

Fig. 7. FTIR spectra of (i) as-synthesized GYSMNP, (ii) GYSMNP after activation with nitric acid, (iii) GYSMNP after grafting with pluronic F-127 (GYSMNP@PF127).

FTIR characterization of GYSMNP@PF127 shows a variety of oxygen-containing functional groups that are favorable for the encapsulation, transport and release of guest molecules, such as chemotherapeutic drugs for drug delivery applications.

(1) *DOX loading studies*

Doxorubicin (DOX) is a cationic chemotherapeutic drug and one of the most commonly used against cancer. However, it presents lethal side effects on healthy cells too. Targeted drug delivery can be an effective method to overcome this issue by delivering the cargo specifically into tumors after being triggered by its abnormal acidic microenvironment. Impressively, the drug loading capacity of GYSMNP@PF127 reached an outstanding 910 $\mu g_{\ DOX}\ mg^{-1}_{\ GYSMNP@PF127}$, which represents a 91% of drug loading efficiency (1:1, w/w). This ability can be ascribed to the π-π stacking interactions between the aromatic rings of DOX and the carbonaceous structure of graphene-based nanoshells, as well as to some surface groups. Besides the π-π stacking, the high DOX loading on GYSMNP@PF127 is also a consequence of the electrostatic interactions and the mesoporous structure of the graphene-based shell.

(2) *Dual pH- and temperature-dependent drug release*

It is well-known that tumor microenvironment presents a weak acidic pH value, around 6.0–7.2, at the tumor tissue due to the Warburg effect, which dramatically decrease to severe acidic pH values, around 4.5–5.5, in tumor endosome/lysosome environment [15]. The pH differences between the tumor microenvironment and normal tissues, pH around 7.4, motivate the development of pH-dependent/responsive nanocarriers that can be specifically designed as smart drug delivery systems triggered by acidic pH values. Therefore, the cumulative pH-dependent DOX release of GYSMNP@PF127-DOX was studied under different pH conditions (7.4, 6.0 and 4.5) either at 37 °C (physiological temperature) or 45 °C (hyperthermia temperature), cf. Fig. 8.

Figure 8 reveals that GYSMNP@PF127 has a pronounced pH- and temperature-dependency drug delivery behavior. The drug release at these conditions takes place in two stages: (I) a rapid DOX release in the first 8 h, followed by (II) a slower rate release till 48 h.

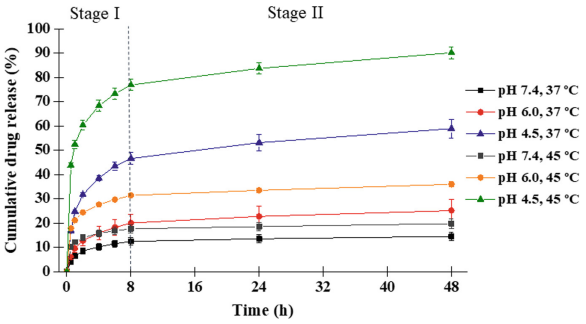

Fig. 8. pH and thermo-responsive release of doxorubicin (DOX) from drug loaded GYSMNP@PF127 under different pH values (7.4, 6.0 and 4.5) and temperatures (37 and 45 °C). Standard deviation of triplicate drug release tests (n = 3).

Once again, the main reasons behind this promising pH/temperature-responsive drug release behavior of the graphene-based material comes from the π-π stacking between the graphene-based nanostructures and the aromatic DOX molecules, which can be easily disrupted under a mild acidic environment [16], but also due to the increased solubility of DOX caused by the protonation process [17]. This will lead to a reduction of the electrostatic attraction between GYSMNP@PF127 and DOX protonated molecules, increasing the progressive drug release.

Overall, graphene-based magnetic nanocarriers shows a great ability to be applied as efficient drug encapsulation and delivery systems, with an especial good pH sensitivity for an intelligent and on-demand drug release on acidic tumor microenvironments.

(3) *Dual pH- and temperature-dependent drug release*

The response of GYSMNP@PF127-DOX as dual pH- and temperature-sensitive drug release nanocarrier was assessed under AMF (f = 340 kHz and H = 21.0 kA m^{-1}) during 30 min, Fig. 9.

Mild hyperthermia temperature (40–43 °C) was reached between 10 and 13 min and maintained until the end of the hyperthermia experiment (30 min) (Fig. 9). The results revealed that after 30 min under AMF, just 6.8% of DOX is released at pH 7.4, while 45.6% at pH 4.5. Therefore, these results suggest that under an AMF, the local heat generated on the GYSMNP@PF127 surface can help the selective diffusion of loaded DOX into the acidic tumor microenvironment through MH. Thus, GYSMNP@PF127 material show high promise to act as dual endogenous pH- and exogenous AMF/temperature-responsive drug nanocarrier for controlled drug delivery as well.

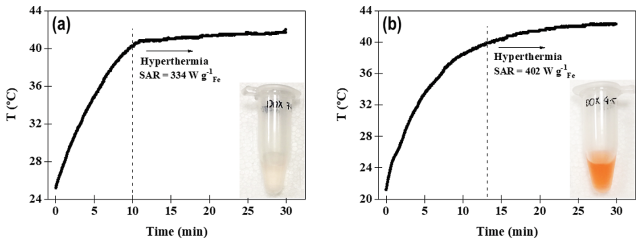

Fig. 9. Magnetic-induced thermal response curve of GYSMNP@PF127-DOX for dual pH- and thermal-responsive drug delivery: (a) dispersed in phosphate buffer at 7.4 to mimic physiological microenvironment, and (b) dispersed in phosphate buffer at 4.5 to mimic tumour microenvironment. Inset shows the drug-containing supernatant resulting from each hyperthermia experiment.

(4) *Cell cytotoxicity assays and cellular uptake of drug nanocarriers*

For a better understanding of the therapeutic effect of the DOX released by the GYSMNP@PF127 material over the tumor cells, HepG2 cells were incubated with the drug nanocarrier for 2, 4 and 6 h (Fig. 10). Cells were observed using confocal microscopy images obtained with Zeiss Laser Confocal Scanning Microscope.

It can be observed (Fig. 10) that the red fluorescence intensity from DOX increases along the incubation time between the nanocarriers and the tumor cells. This observation reveals that the DOX is continuously released from GYSMNP@PF127-DOX nanocarriers. After 6 h, the cell nuclei exhibit a strong DOX red fluorescence intensity, suggesting that a high content of DOX is released into the cell cytoplasm and reaches the nucleus after nanoparticle internationalization by endocytosis. The cellular uptake of drug nanocarriers is not only very important for enhancing the drug delivery efficiency, but it is also beneficial for magnetic hyperthermia therapy, allowing a combinatorial thermo-chemotherapeutic effect.

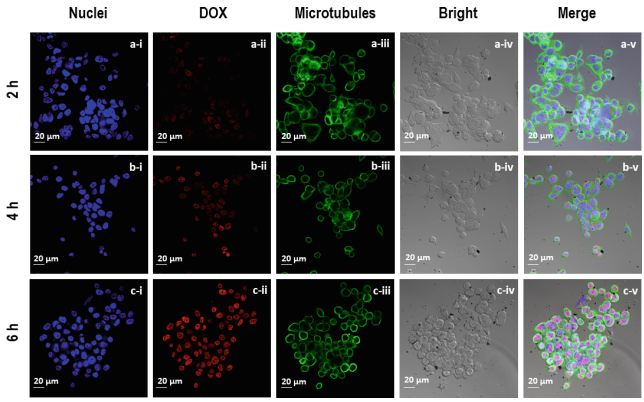

Fig. 10. Confocal microscopy images of HepG2 cells incubated with GYSMNP@PF127-DOX at a concentration of 5 μg mL^{-1} during 2 h (a), 4 h (b) and 6 h (c). (i) Cell nuclei stained with Hoechst and excited at 405 nm; (ii) DOX released in HepG2 cells and excited at 561 nm; (iii) Cell microtubules immunolabeled with the monoclonal anti-tubulin antibody (B512) combined with a secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 488 excited at 488 nm; (iv) HepG2 cells using bright contrast; (v) Cell merged images.

This combined strategy represents an important step forward in the fight against cancer and highlight this new generation of responsive graphene-based nanocarriers as suitable therapeutic actuators on it.

The compilation of this study was published in the journals *C – Journal of Carbon Research* [18], and *Material Science Engineering C* [19].

4 Dual-Organ-on-a-Chip Platform Developed for Drug Screening Studies in Nanomedicine

Organ-on-a-chip platforms are novel microfluidic tools that mimic complex human organ functions at the microscale level and predict with high accuracy human responses to drugs and theranostic agents. Herein, a novel dual organ-on-a-chip platform integrated with multiplexed electrochemical analysis is described to evaluate with high accuracy the performance of new drug nanocarriers, such as the developed GbMNP, as intelligent and on-demand drug delivery system with pH-dependent controlled release.

This work was developed under the framework of a Fulbright Research Grant 2017, at the Harvard-MIT Division of Health Sciences and Technology (Cambridge, Massachusetts, USA), headed by Prof. Ali Khademhosseini and supervised by Dr. Su Ryon Shin. This bioengineering leading group has a vast experience in developing organ-on-a-chip systems that aims to mimic human response to various chemicals.

4.1 Dual Organ-on-a-Chip

A dual organ-on-a-chip platform, compressing cardiac and breast cancer organoids, with the aim to evaluate the effect of chemotherapy over those organs, in continuous and during several days is shown in Fig. 11. Due to the versatility of the proposed 3D tissue/organ platform, the evaluation of novel drug nanocarriers, such as the GYSMNP@PF127-DOX developed in Sect. 3, is explored in contrast with free chemotherapy.

The dual organ-on-a-chip platform was obtained by embedded cardiac iPSCs (healthy cardiac model) and SkBr3 spheroids (breast cancer model) inside a gelation methacryloyl (GelMA) hydrogel photo-cross linked, generating the human cardiac- and breast cancer-organ-on-a-chip bioreactor (Fig. 12).

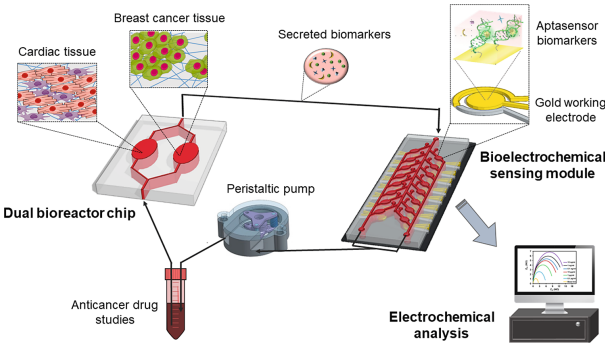

Fig. 11. Schematic representation of the feedback loop of dual organ platform (comprising breast cancer and cardiac organoids) integrated with multiplexed electrochemical analysis for the chemotherapy study effect of doxorubicin using the free or encapsulated drug.

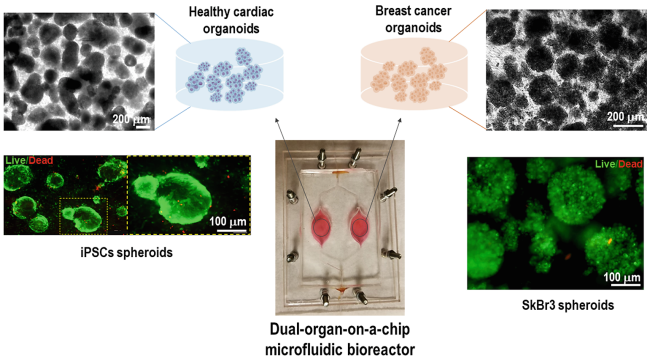

Fig. 12. Schematic representation of the dual organ-on-a-chip microfluidic bioreactor developed to monitor the effect of the chemotherapeutic drug DOX (free or encapsulated in nanocarriers) over the breast cancer and cardiac healthy organoids. Life/dead confocal images from iPSCs and SkBr3 spheroids showing the high viability of these organoids inside the GelMA hydrogel after UV photo-cross linking. Live cells stained green and dead cells stained red.

The development of the biomimetic dual organ-on-a-chip system allows the simultaneous monitoring and investigation of the effect of chemotherapeutic drugs over aggressive breast cancer (such SkBr3 cell line) and healthy cardiac tissues. This non-invasive continuous monitoring of multi-organ systems offers the possibility to obtain new insights over the cellular metabolic activity, as well as to develop new drug models or adjust chemotherapeutic doses in a personalized manner. To date, and to the best of our knowledge, this was the first study developed to determine the effect of anticancer drugs over multi-organs-on-a-chip platforms using biomolecular sensing (being prepared to publication).

4.2 A Label-Free Electrochemical (EC) Sensing Method

The immobilization process of the selected aptamers in the biosensors was performed as described elsewhere [20]. Two cardiac biomarkers (CKMB and Troponin T), and one breast cancer biomarker (HER2) were exploited.

Aptamers, which are short single-stranded oligonucleotides capable of binding molecules with high affinity and specificity, were used in this study to target specific antigens (troponin-T, CKMB and HER2), in a highly reproducible manner (Fig. 13).

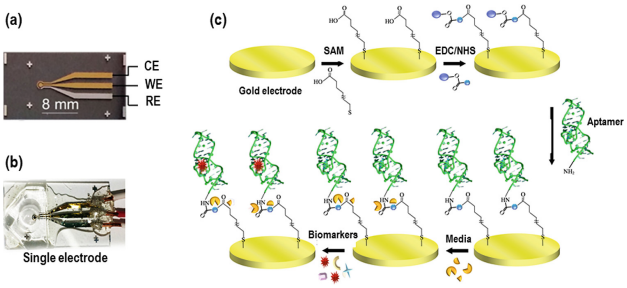

Fig. 13. (a) Image of the microfabricated electrode set containing a reference electrode (RE), a counter electrode (CE) and a working electrode (WE); (b) image of a single electrode as the one used in this study; (c) schematic diagram of the immobilization steps of the aptamers into the microelectrode.

4.3 Results and Discussion

Samples collected from drug-induced experiments along 5 days were measured in individual aptamer-based biosensor for each biomarker by electrochemical impedance spectroscopy (Fig. 14(b–d)). As expected, free DOX shows to induce a dramatic toxicity over the breast cancer tissue, as revealed by the decrease of HER2 biomarkers along the 5 days of chemotherapeutic treatment, but also reveals cardiac injury to the heart organoid with increasing detection of both cardiac diseases' biomarkers (i.e., Troponin-T and CKMB). On the other hand, the encapsulated DOX released by the nanocarrier GYSMNP@PF127-DOX shows a more protective behavior over the healthy cardiac organoids in comparison with the free DOX, and at the same time maintaining the severe

toxicity over the breast cancer spheroids. These exciting results attest the previous screening results detailed in Sect. 3, where the developed graphene-based magnetic nanoparticles were tested as smart and on-demand drug nanocarriers triggered by the abnormal acidic pH found in tumor microenvironment.

In addition to the aptamer-based biosensing analysis, the cell viability of the cardiomyocytes and breast cancer organoids after the chemotherapeutic treatment (day 5) were analyzed by live/DOX assay (cf. Fig. 15).

The confocal images subscribe the results obtained from the aptamer-based biosensing analysis, showing that free DOX is delivered in the same proportion on both organoids (healthy cardiac tissues and breast cancer), which cause the damage of cardiac tissues and the release of the cardiac biomarkers (Troponin-T and CKMB). On the other hand, DOX encapsulated in the graphene-based nanocarriers shows high-target delivery over the breast cancer organoids compared to cardiac spheroids, as ascribed by the low amount of cardiac biomarkers detected with the biosensor system. The results confirm the potentiality of the developed GYSMNP@PF127 material as smart and on-demand drug nanocarriers for the treatment of cancer, and the extraordinary ability to use the aptamer-based electrochemical biosensor to detect trace amounts (pg mL^{-1}) of several biomarkers secreted from multi-organ systems, which are undetectable by using conventional methods, such as ELISA (ng mL^{-1}).

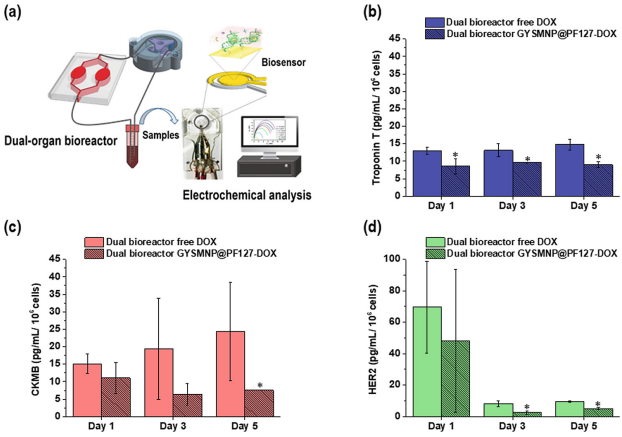

Fig. 14. (a) Schematic representation of the feedback loop system used during the drug induced experiments on dual organ-on-a-chip microfluidic bioreactor along 5 days. Electrochemical sensing of selected biomarkers released from dual organ-on-a-chip bioreactor: (b) cardiac biomarker Troponin-T; (c) cardiac biomarker CKMB; (d) breast cancer biomarker HER2. Error bars show standard deviation of individual single microsensors (n = 3) and the asterisks (*) represent statistical significance in comparison with free DOX assay for $p < 0.05$ determined by Student's t-test.

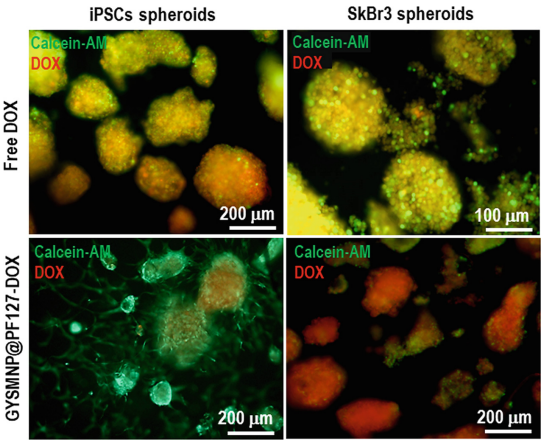

Fig. 15. Cell viability assays of cardiomyocytes (iPSCs) and breast cancer (SkBr3) spheroids obtained after 5 days of chemotherapeutic treatment (free or encapsulated DOX) in dual organ-on-a-chip bioreactors. Live cells stained with green Calcein-AM dye and red cells showing DOX uptake.

5 Conclusion and Future Perspective

The main objectives of this work were the development of carbon-based magnetic nanoparticles suitable for nanomedicine and their microfluidic study using new bio-platforms, aiming new insights over the complex biophysical and toxicological interaction of these nanosystems with human cells (i.e. blood, healthy and tumor cells). The successful achievement of these objectives is involved in complex multidisciplinary approaches, namely chemistry, physics, materials science, biology, bioengineering and biomechanics, which were accomplished by subdividing these objectives in subsequent tasks.

Overall, the developed carbon-based nanosystems, as well as the proposed microfluidic platforms, described in this work, allows to contribute to a better understanding of the biophysical and biological interaction of new biomedical nanosystems designed for nanomedicine.

It was shown that the evolution of smart and multifunctional nanomaterials in nanomedicine could be extremely beneficiated by the parallel evolution of new microfluidic platform model systems to study their safety, bio-distribution and efficacy.

On this demand, organ-on-a-chip platforms can have a significant impact in this field. There are still many challenges to be addressed on the development and optimization of these advanced 3D microfluidic platforms, but the potential and possibilities are far more enthusiastic. Therefore, and as predicted by Professor Langer and Khademhosseini, pioneers in drug delivery and tissue engineering [21]: “with sufficient time and research the promise of nanotechnology based medicine may become a reality”.

Acknowledgment. The successful accomplishment of the multidisciplinary tasks considered in this Ph.D. work, was supported by important collaborations that were strengthened at different stages in the frame of this Ph.D. project, namely INL - International Iberian Nanotechnology Laboratory (Braga, Portugal); CeRiCol - Centro Ricerche Colorobbia Consulting (Vinci, Italy); CIMO – Centro de Investigação da Montanha (Bragança, Portugal) and Harvard-MIT Division of Health Sciences and Technology (Cambridge, USA).

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