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# Encapsulation of $VEGF_{165}$ in magnetic PLGA nanocapsules for potential local delivery and bioactivity into human brain endothelial cells

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**Abstract:**

Angiogenesis is an important repairing mechanism in response to ischemia. The administration of pro-angiogenic proteins is an attractive therapeutic strategy to enhance angiogenesis after an ischemic event. Their labile structures and short circulation times *in vivo* are the main obstacles that reduce the bioactivity and dosage of such proteins at the target site. We report on poly(D,L-lactic-*co*-glycolic acid) (PLGA) nanocapsules (diameter < 200 nm) containing bioactive vascular endothelial growth factor-165 ($VEGF_{165}$) in the inner core and superparamagnetic iron oxide nanoparticles (SPIONs) embedded in the polymeric shell. The system showed good encapsulation efficiencies for both$VEGF_{165}$ and the SPIONs and a sustained protein release over 14 days. *In vitro*

studies confirmed protein bioactivity in the form of a significantly increased proliferation in human microvascular brain endothelial cell cultures once the protein was released. Through magnetic resonance imaging (MRI) measurements we demonstrated excellent $T_2$ contrast image properties with $r_2$ values as high as 213 $mM^{-1}$ $s^{-1}$. In addition, magnetic $VEGF_{165}$-loaded PLGA nanocapsules could be displaced and accumulated under an external magnetic field for guiding and retention purposes. We therefore suggest that $VEGF_{165}$-loaded magnetic PLGA nanocapsules may become a new targeted protein-delivery strategy in the development of future pro-angiogenic treatments, as for instance those directed to neurorepair after an ischemic event.

## Introduction

Angiogenesis, the formation of new blood vessels from pre-existing ones, is an important repairing mechanism in response to ischemia. It is increasingly being established that angiogenesis enhancement, after an ischemia event, facilitate patient recovery. The administration of pro-angiogenic proteins is an attractive therapeutic strategy to enhance local angiogenesis[1,2]. Several approaches are under investigation to achieve efficient and non-invasive local angiogenic treatments[3]. One of the most frequently used approaches consists of encapsulating pro-angiogenic proteins into suitable polymeric micro-[4,5,6] or nano-carriers[7,8] which may preserve protein structure and allow local delivery with reduced off-target effects. Recombinant human vascular endothelial growth factor-165 ($VEGF_{165}$) is one of the most studied pro-angiogenic growth factors[9] and it has been encapsulated with high encapsulation efficiencies into poly(D,L-lactic-*co*-glycolic acid) (PLGA) microspheres[5] and nanocapsules[7,10]. Importantly, evidence of angiogenesis induced by the delivery of $VEGF_{165}$ from PLGA carriers has also been provided by *in vivo* assays, which show tissue revascularization and recovery in animal models of hind-limb ischemia[11,12,13].

The use of magnetic materials to assist on-site delivery of therapeutic agents, with an external magnetic field, has raised considerable interest. There have been several studies on the encapsulation of superparamagnetic iron oxide nanoparticles (SPIONs) into PLGA particles[14,15,16] together with drugs (*e.g.*, anti-cancer and anti-arthritis molecules), for targeted drug delivery purposes and for use in non-invasive imaging (by magnetic resonance imaging, MRI) of tissues[17,18,19,20,21]. On the other hand, there are only a few examples of the co-encapsulation of SPIONs with proteins[22,23] and to date, VEGF has only been encapsulated into PLGA particles with gadolinium complexes[24]. The lack of published research on protein–SPION co-encapsulation could be related to the labile structure of proteins and to their potential inactivation after adsorption on the surface of SPIONs[25]. In fact, protein adsorption may induce conformational changes of the native structure with the exposure of novel epitopes that may transmit different biological signals[26].

Here, we report on the co-encapsulation of recombinant human $VEGF_{165}$ and SPIONs using a double emulsion-solvent evaporation method. In this process, $VEGF_{165}$ has been encapsulated in the inner core of a PLGA nanocapsule, whilst hydrophobic oleic-acid-coated SPIONs have been embedded in the organic polymer phase. Our purpose is to demonstrate that, by confining $VEGF_{165}$ and SPIONs in two different compartments of the carrier, protein bioactivity is preserved and that the magnetic nanocapsules can be externally guided in presence of a suitable magnetic field.

## Materials and Methods

### Nanocapsule synthesis

In a small propylene tube, an organic phase containing PLGA and SPIONs was prepared as follows: 50 mg of PLGA 50:50 (RG502 Boehringer Ingelheim, *Mw* = 18000 Da, inherent viscosity 0.16–0.24 dL $g^{-1}$) were mixed with 0.45 mL of dichloromethane

(Sigma Aldrich) by using an ultrasonic bath. Oleic-acid-coated SPIONs (0.94 mg) were dispersed in 50 μL of dichloromethane and then mixed with the polymer solution in an ultrasonic bath.

25 μg of lyophilized recombinant human $VEGF_{165}$ (Peprotech) dissolved in 50 μL of EBM (endothelial basal medium, Lonza) were added in one drop to the organic phase. The first emulsion was obtained by sonicating at 240 W for 28 s (Vibra-cell® VCX 500, Sonics & Materials). The temperature during the whole emulsion process was kept at 4 °C by use of an ice bath. The second emulsion was prepared by adding 2 mL of 2% w/v polyvinyl alcohol (PVAL, Sigma Aldrich) aqueous solution and sonicating for additional 28 s. The as-formed colloidal suspension was poured into 50 mL of MilliQ water and kept under mechanical stirring at room temperature for 2 h to allow complete evaporation of the organic solvent. Then the colloidal dispersion was washed with MilliQ water three times with centrifugations at 9469 × *g* (centrifuge Alegra® 64R Beckman), each time with addition of fresh water, to reduce the amount of PVAL adsorbed on the PLGA. Finally, the particles were re-dispersed in an aqueous solution containing 0.2% w/v trehalose (Sigma Aldrich) before freeze-drying at –80 °C for 2 days. The as-obtained powder was stored at 4 °C with desiccant silica gel.

**Experimental methods**

Dynamic light scattering (DLS)

Particle hydrodynamic diameter was measured by re-dispersion of 0.6 mg of lyophilized powder into 1 mL of MilliQ water. Measurements were performed with a Zetasizer Nano ZS from Malvern Instruments equipped with a He/Ne 633 nm laser using a disposable plastic cuvette. Size measurements were run in triplicate, each for 15 scans, at 25ºC. Zeta potential measurements were run three times at 25ºC.

Scanning electron microscopy (SEM)

2 mg of the lyophilized powder were re-dispersed into 600 μL of MilliQ water and centrifuged twice at 1073 × *g* for 20 min. Each time the supernatant was discarded and fresh water added. Finally, particles were re-dispersed in 1 mL of water and one drop of the slightly turbid suspension was deposited onto a small slice of silicon wafer stuck on top of a carbon layer. The sample was dried at room temperature and then covered with 2 nm of Au–Pd (Emitech K550 Sputter Coater, 25 mA for 1 min). Images were acquired under high vacuum, with a FESEM Merlin, Zeiss using frame-scan modality, repeated 15 times.

Cryo-transmission electron microscopy (Cryo-TEM)

Cryo-TEM experiments were performed on aqueous suspensions of nanoparticles at a concentration of 1 mg $mL^{-1}$. After one washing with MilliQ water by using a centrifugation at 1073 × *g* for 20 min (centrifuge Minispin®, Eppendorf®), a drop of suspension was placed onto a Quantifoil® grid where a perforated foil was used to bear an ultra-thin carbon support foil to minimize the total specimen thickness. The drop was blotted with filter paper and the grid was quenched rapidly into liquid ethane to produce vitreous ice, avoiding the formation of crystals. The grid was then transferred into the TEM microscope (JEM-2011 operating at 200 kV), where the temperature was kept under –140 °C by use of liquid nitrogen during the imaging.

Superconductive quantum interference device (SQUID)

A magnetometer from Quantum Design MPMS5XL was used to perform magnetization measurements of SPIONs. A gelatin capsule was filled with 3 mg of lyophilized powder together with some cotton to reduce sample movement during measurements with applied magnetic field. The as-prepared sample was inserted into the SQUID magnetometer

sample holder. The amount of encapsulated SPIONs was evaluated as follows: initially, the remnant magnetization value of the magnetized nanocapsules ($M_{R\ nanocapsule}$) was measured at 5 K after the material was magnetized at 5 T and this value (emu) was divided by the weight of the analyzed powder (emu/ $g_{nanocapsule}$). Subsequently, the remnant magnetization per gram of nanocapsule (emu/$g_{nanocapsule}$) was divided by the remnant magnetization per gram of SPIONs (emu/$g_{Fe2O3}$) measured at the same conditions giving the average amount of magnetic material inside a nanocapsule ($g_{Fe2O3}$/ $g_{nanocapsule}$). The as-obtained "experimental SPION loading" was divided by the "nominal SPION loading" to calculate SPION encapsulation efficiency, as follows:

$$EE\% = \frac{experimental\ SPIONs\ loading}{nominal\ SPIONs\ loading} \times 100$$

Magnetic resonance imaging (MRI)

Phantoms were prepared by filling 2.5 mL microtubes with a solution of 1.5% agarose in water, into which different amounts of nanocapsules had been admixed. Such a set of phantoms spanning the iron dose range from 0 to 15 μg $mL^{-1}$ was prepared with four different batches of synthesized nanocapsules. Agarose phantoms were kept at 4 °C until imaged in a 9.4 T MR system (BioSpin 94/30, Bruker BioSpin, Ettlingen, Germany) by using a quadrature 86 mm inner-diameter volume coil as follows: To determine $T_2$ relaxation times in each phantom, a series of $T_2$ weighted images were acquired with a multi-slice multi-echo (MSME) sequence, with TR = 3 s, 30 TE values from 11 to 330 ms (11 ms echo spacing), matrix size = 256 × 256, field of view (FOV) = 65 × 65 mm, and slice thickness = 1.5 mm (four adjacent slices). Quantitative $T_2$ values were obtained from hand-drawn regions of interest by using curve fitting in the Image Sequence Analysis (ISA) Tool (ParaVision v.5.1, Bruker BioSpin, Ettlingen, Germany).

Total $VEGF_{165}$ determination by enzyme-linked immunosorbent assay (ELISA)

Low-weight Eppendorf pipettes (Eppendorf®) were used for the following experiments: Three independent batches of nanocapsules loaded with $VEGF_{165}$ and SPIONs were analyzed simultaneously with PGLA nanocapsules loaded with SPIONs only (control). Lyophilized nanocapsules (5 mg) were previously sterilized under UV rays for 18 h[27]. To determine $VEGF_{165}$ encapsulation efficiency, nanocapsules were dissolved at a concentration of 10 mg $mL^{-1}$ in an aqueous solution of 0.1 M NaOH, 10% dimethyl sulfoxide (DMSO), 0.2% w/v sodium dodecyl sulfate (SDS), under magnetic stirring[28]. The as-obtained samples were centrifuged at 13226 × *g* for 5 min to precipitate SPIONs. The collected supernatants were stored at –80 °C until used to determine total $VEGF_{165}$ content by ELISA (Quantikine® ELISA human VEGF immunoassay, catalog number DVE00, R&D Systems).

In parallel, to measure the amount of $VEGF_{165}$ released from nanocapsules, sterilized lyophilized samples (5 mg from each batch) were dispersed at a concentration of 10 mg $mL^{-1}$ in a release medium consisting of 0.1% w/v bovine serum albumin (BSA) and 1% penicillin–streptomycin in phosphate-buffered saline (PBS 1X). Subsequently, all samples were placed in a water bath (37 °C) with horizontal shaking (80 oscillations per minute). Aliquots of 0.1 mL were taken after 1 and 6 h, 2, 6, and 14 days, centrifuged at 13226 × *g* for 5 min and the supernatant was stored at –80 °C until used for measuring $VEGF_{165}$ content by the Quantikine® ELISA (R&D Systems). The volume of the aqueous suspensions was kept constant during the release study by adding fresh release medium.

$VEGF_{165}$ concentration (pg $mL^{-1}$) was measured by colorimetric absorption at wavelengths of 450 nm and 540 nm. All samples were run in duplicate and mean values were used (coefficient of variation between duplicates was less than 20%).

*In vitro* $VEGF_{165}$ bioactivity

VEGF-containing and control samples obtained during the release study were used to assess protein bioactivity. Briefly, supernatants stored at –80ºC were thawed and five samples corresponding to the different times of analyzed release (1 and 6 h, 2, 6, and 14 days), were obtained by pooling three batches (RG502_ 23, 24, and 25, see Table 1). Supernatants of VEGF-free nanocapsules (batch RG502_27) collected at different times were used as the control medium.

Human cerebral microvascular endothelial cells (hCMEC/D3)[29] were used to assess the activity of encapsulated and released VEGF by measuring cell proliferation and viability. Briefly, $2.5 \times 10^3$ cells were seeded in 24-well-plates in endothelial cell growth media, (EGM-2, Lonza) containing endothelial basal media (EBM) plus supplements: VEGF, insulin-like growth factor, bovine FGF, hydrocortisone, ascorbate, and 2% fetal bovine serum (FBS). After four days in culture, cells were thoroughly washed with phosphate-buffered saline (PBS 1X) and treated with pooled VEGF media to a final concentration of 10 ng $mL^{-1}$ of VEGF (according to the ELISA results). This concentration has been shown by other authors to induce *in vitro* proliferation of endothelial cells[30]. Two controls were run in each experiment: EBM and medium of batch RG502_27 (release control). EBM was used to load all wells to a final volume of 0.4 mL. After 48 h of treatment cells were washed, trypsinized, and the number of total cells and number of viable cells were counted with the Muse™ Cell Count and Viability Kit, as described[31]. Each experiment was run in duplicates in three independent experiments. The mean value of each independent experiment was used for statistics which consisted of one-way ANOVA and Dunnett *post hoc* test (SPSS 15.0 software was used). Proliferation data is expressed as percentage of control condition (basal media).

## Results and Discussion

Proteins are attractive therapeutic molecules but are sensitive to changes in their environment (pH, temperature, ionic force, *etc*.). Pre-clinical studies, using PLGA nanocapsules loaded with pro-angiogenic factors, have demonstrated great potential in ischemic treatments by inducing a more efficient revascularization compared to administration of the protein alone[4,32]. We selected $VEGF_{165}$ as a pro-angiogenic factor that activates endothelial cells by inducing cell proliferation and migration[33]. We encapsulated $VEGF_{165}$ into PLGA nanocapsules along with SPIONs, with the aim to improve the accumulation of bioactive protein at a target site under a magnetic field gradient. In fact, despite several studies on the formulation of $VEGF_{165}$ loaded PLGA nanocapsules suitable for intravenous administration[7,10,34], very few efforts have been done to achieve local delivery of $VEGF_{165,}$ since its distribution in non-targeted tissues may cause unwanted side-effects[35,36].

The size of the PGLA nanocapsules was measured by re-constitution of the freeze-dried material in MilliQ water (Table. 1). The zeta potential of particles in water was –22 ± 2 mV. Particles could be readily re-dispersed in water (Fig. 1) and the suspension was stable without precipitation over a period of three months. Importantly the hydrodynamic size and polydispersity of four different batches were reproducible; the average calculated over five batches was 223 nm with a standard deviation of 10 nm. Nanocapsule size was measured by counting 200 particles by SEM. The average diameter was 165 nm with 20% polydispersity (Fig. 2), which is considered to be suitable for intravenous administration[37].

Cryo-TEM confirmed that SPION encapsulation had occurred, with uniform magnetic nanoparticle distribution (visible as black spots in Fig. 3a) within the polymeric matrix. Moreover, the analysis showed a vitrified aqueous dispersion of PLGA nanocapsules, which reflect the administration state, with well-defined spherical shape and no particle

aggregation (Fig. 3a). Magnetic measurement at 5 K of lyophilized powder showed high saturation magnetization of nanocapsules at around 80 emu $g^{-1}$ $Fe_2O_3$, a critical parameter in view of magnetic retention (Fig. 3b). Moreover zero-field cooling, field cooling analysis (ZFC-FC) demonstrated the superparamagnetic character of the nanocapsules, and that SPIONs did not aggregate during the encapsulation process since the blocking temperature ($T_B$) of the nanocapsules (44 K) was very close to the $T_B$ value of SPIONs in aqueous dispersion (46 K; inset Fig. 3b). We have therefore demonstrated that SPIONs are homogenously dispersed in the polymer matrix, retaining their superparamagnetic behaviour after the encapsulation process. This is an important result, because it indicates the absence of magnetic dipoles and attractive interactions among nanocapsules that would cause embolism during the intravenous administration. Furthermore, our magnetic nanocapsules present a high relaxivity value and they can be used as contrast agents in magnetic resonance imaging (MRI).

The starting amount of $VEGF_{165}$ used for PLGA encapsulation is usually between 0.03 and 0.1% w/w[10,38] and the encapsulation efficiency (EE%) is around 70% for particles of 300 nm of hydrodynamic diameter[10]. For the encapsulation of SPIONs into PLGA nanocapsules, the achieved final concentration was 1.4% w/w[16] in particles of around 200 nm in diameter. In our experiments, the initial $VEGF_{165}$ loading was 0.05% w/w, and SPION loading was 1.8% w/w (expressed as iron weight over PLGA mass used for the encapsulation). We achieved a VEGF EE% value of up to 58% and a SPION EE% value of up to 68% (Table 1), which are both in good agreement with the literature values[10,16].

With a simple experiment, we checked the magnetic retention of the nanocapsules by application of an external magnetic field. We placed an FeNdB permanent magnet (0.4 T) 3 mm away from the colloidal magnetic suspension contained in an Eppendorf tube and after 24 h, we saw retention of the nanocapsules on the plastic wall close to the magnet (Fig. 4). This experiment was a "proof of concept" of the potential of $VEGF_{165}$- and

SPION-loaded PLGA nanocapsules' use as magnetic guiding tools to achieve targeted drug delivery. Previous experiments that used microparticles loaded with only 1% w/w iron oxide, demonstrated a magnetic retention effect in rodents' knees, which suggests that our VEGF magnetic nanocapsules may have adequate properties for magnetic targeting[39], considering that our average amount of loaded iron oxide is 1.16% ± 0.8 (n=5).

From MRI measurements, we found optimal $T_2$ relaxation properties of SPION-loaded PLGA nanoparticles with an average transverse relaxivity value ($r_2$) of 181 ± 37 $mM^{-1}$ $s^{-1}$, calculated over four different batches (Table 2). Figure 5 shows $T_2$ weighted phantoms with hypointense signal decay in a concentration-dependent manner. Such high relaxivity value confirms the theranostic character of our nanocapsules, suggesting that it would be possible to track them *in vivo* by MRI.

Protein release experiments resulted in a sustained $VEGF_{165}$ release with almost 35% of the total cargo discharged over a period of two weeks; the process was faster during the first hours (Fig. 6).

Finally, we demonstrate that recombinant human $VEGF_{165}$ remained bioactive after the encapsulation and subsequent release process. We used the collected media from the $VEGF_{165}$ release study to prove human cerebral microvascular endothelial cell proliferation *in vitro*. Figure 7 shows that the number of cells, treated with release media from $VEGF_{165}$ and SPION-loaded PLGA nanocapsules, was significantly increased compared to control medium (SPION-loaded nanocapsules without $VEGF_{165}$) and basal medium. The highest cell proliferation occurred with release media collected at 1 and 6 h, compared to both control conditions. Release media obtained at 2, 6, and 14 days also induced cell proliferation, although to a lesser extent. This decrease in bioactivity might be explained by partial protein inactivation over time in acidic pH which arises from hydrolytic degradation of PLGA, or by the short protein half-life at 37ºC.

Cell viability was not affected by our treatments since all conditions presented a mean number of viable cells over 90%. In particular, viability percentages *vs*. total cells were 94 ± 2 for basal media, 94 ± 1 for control nanocapsules without VEGF, 94 ± 2 for VEGF release at 1 h, 96 ± 1 for VEGF release at 6 h, 93 ± 1 for VEGF release at 2 days, 94 ± 1 for VEGF release at 6 days, and 92 ± 2 for VEGF release at 14 days.

## Conclusions

By means of a double-emulsion–solvent-evaporation method, we synthesized PLGA nanocapsules which contained human recombinant $VEGF_{165}$ in their inner particle core and SPIONs embedded in their polymeric layer. The nanocapsules were spherical, very monodisperse, with a hydrodynamic diameter of around 220 nm that is suitable for intravenous administration and good encapsulation efficiencies of both $VEGF_{165}$ and SPIONs. Importantly, the particles were magnetically retained under an applied magnetic field gradient, showed sustained protein release over time and *in vitro* pro-angiogenic activity by inducing human microvascular brain endothelial cell proliferation. We suggest that the co-encapsulation of $VEGF_{165}$ and SPIONs in different compartments of the same formulation (PLGA nanocapsule) may be a promising strategy for developing new targeted therapeutic treatments to enhance angiogenesis.

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**Table 1**: Characterization of samples synthesized by double emulsion-solvent evaporation method, containing $VEGF_{165}$ and SPIONs (from RG502_23 to RG502_25) and just with SPIONs (RG502_26, 27). $VEGF_{165}$ amount in PLGA nanocapsules was measured by means of ELISA immunoassay. SPION content was determined by SQUID measurements. EE%= entrapment efficiency %; NCs= nanocapsules.

| SAMPLE | PROTEIN | PROTEIN EE% | PROTEIN/NCs ($ng \cdot mg^{-1}$) | SPIONs EE% | SPION/NCs ($\mu g \cdot mg^{-1}$) | SYNTHESIS YIELD% | DIAMETER (DLS, nm) |
|---|---|---|---|---|---|---|---|
| RG502_23 | VEGF | 58% | 279 | 60% | 10.8 | 62% | 235 (17% PD) |
| RG502_24 | VEGF | 57% | 273 | 60% | 10.8 | 63% | 225 (17% PD) |
| RG502_25 | VEGF | 44% | 211 | 68% | 12.2 | 68% | 230 (18% PD) |
| RG502_26 | _ | _ | _ | 57% | 10.3 | 65% | 215 (17% PD) |
| RG502_27 | _ | _ | _ | 65% | 11.7 | 63% | 210 (15% PD) |

**Table 2**: $r_2$ ($mM^{-1}$ $s^{-1}$) values obtained by MRI analysis of lyophilized SPIONs and $VEGF_{165}$-loaded PLGA nanocapsules in agarose. Samples RG502_23, 24, and 25 contain $VEGF_{165}$ and SPIONs. Sample RG502_27 contains only SPIONs.

| Sample | $r_2$ ($mM^{-1}$ $s^{-1}$) |
|---|---|
| RG502_23 | 180 |
| RG502_24 | 130 |
| RG502_25 | 201 |
| RG502_27 | 213 |

**Figure 1**: DLS measurements of as-synthesized nanocapsules in water (before lyophilization) with mean diameter of 225 nm and 17% polydispersity (black full circles); nanocapsules after freeze-drying and re-dispersion in water with mean diameter of 256 nm and 18% polydispersity (black empty circles).

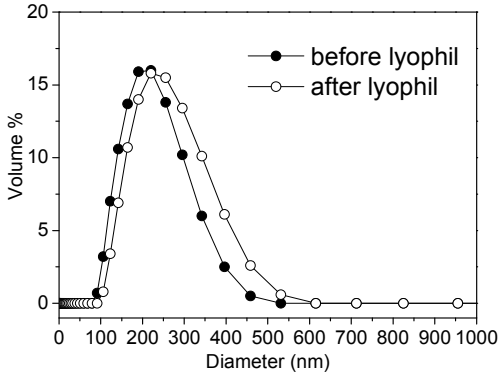


**Figure 2**: SEM image of PLGA nanocapsules synthesized by double emulsion-solvent evaporation method, with inset showing the empty inner core of a nanocapsule. Scale bar in the inset of 100 nm.

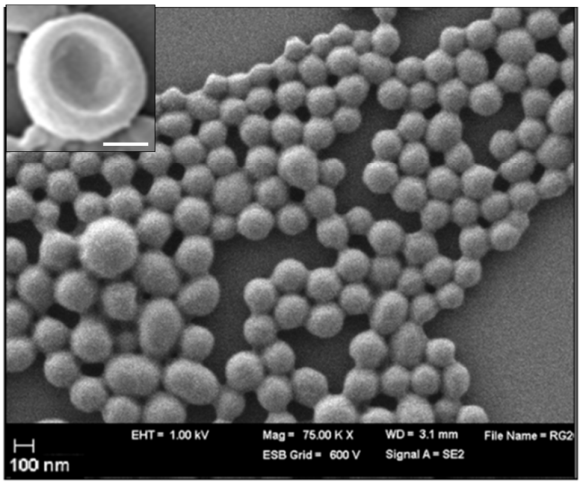


**Figure 3:** SPIONs embedded in PLGA nanocapsules: a) Cryo-TEM images of PLGA nanocapsules dispersion in water. Images show spherical nanocapsules with SPIONs visible as black spots, uniformly dispersed in the polymer matrix; b) magnetic measurement of a lyophilized batch RG502_23 at 5 K, showing high saturation magnetization (78 emu $g_{Fe2O3}^{-1}$). ZFC-FC measurement (inset) with a blocking temperature of 44 K.

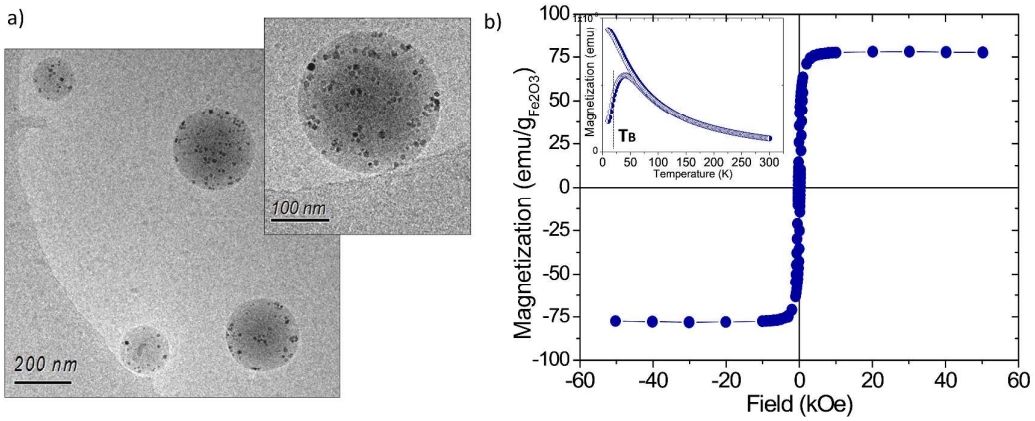


**Figure 4:** Eppendorf with 0.2 mL of SPION-loaded PLGA nanocapsules at a concentration of 17 mg $mL^{-1}$. A strong magnet (dimensions 5 × 5 × 2 cm, field 0.4 T) was placed near the Eppendorf. After 24 h, particles adhered to the wall close to the magnet.

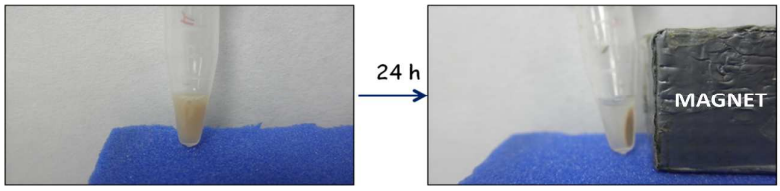


**Figure 5:** $T_2$-weighted phantoms obtained by mixing lyophilized SPION-loaded PLGA nanocapsules with agarose at increasing iron concentrations. The image corresponds to phantoms of sample RG502_23.

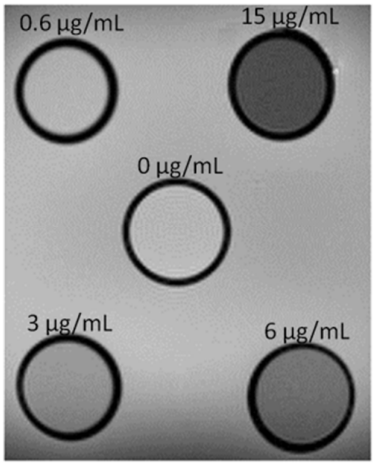


**Figure 6**: Protein release curve over a period of two weeks for nanocapsules loaded with $VEGF_{165}$ and SPIONs (ELISA immunoassay). The release is expressed as the average of three samples analyzed at the same time; error bars indicate standard deviation. Aliquots were taken after 1 and 6 h, 2, 6, and 14 days.

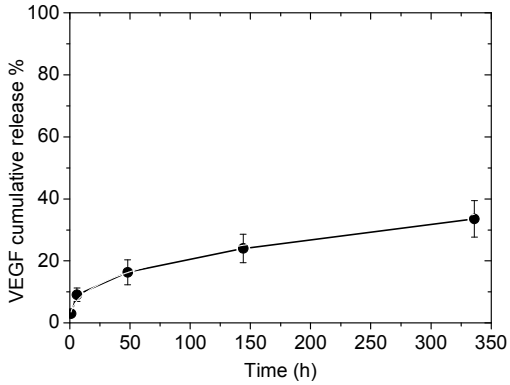
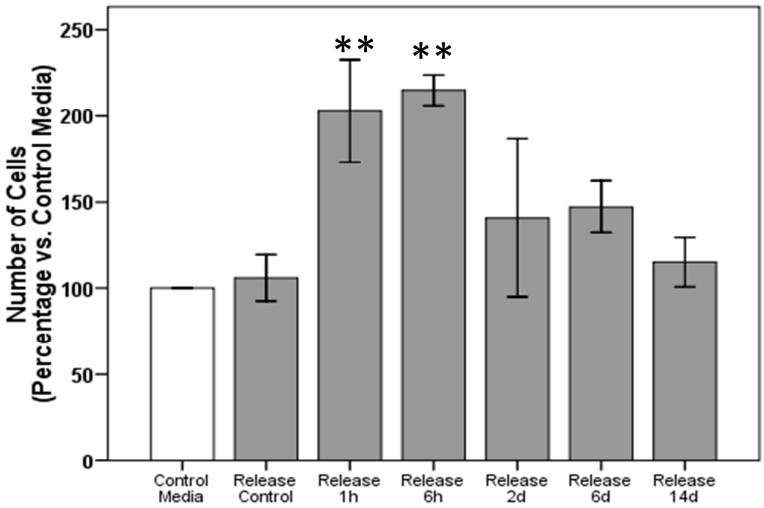


**Figure 7**: Bar graphs representing the percentage of endothelial cells in each treatment condition *vs*. control treatment (basal media). Bars represent mean ±SD of n=3 independent experiments. ** p<0.001.



## Table of contents

New drug delivery systems based on biodegradable magnetic nanocapsules for targeted delivery of pro-angiogenic proteins, potentially useful in therapeutic angiogenesis.

## Graphical Abstract