Magnetically responsive biopolymeric multilayer films for local hyperthermia

M. Criado, B. Sanz, G. F. Goya, C. Mijangos and R. Hernández

We present a proof of concept on the use of thermomagnetic polymer films (TMFs) as heating devices for magnetic hyperthermia in vitro. The TMFs were prepared through spray assisted layer-by-layer assembly of polysaccharides and magnetic iron oxide nanoparticles, yielding an alternate magnetic-polymer multilayer structure. By applying a remote alternating magnetic field (AMF) \( f = 180 \text{ kHz}; H = 35 \text{ kA m}^{-1} \) we increased the temperature of the TMFs in a thickness-dependent way, up to 12 \( ^\circ \text{C} \) within the first 5 minutes. To test our films as heating substrates for magnetic hyperthermia, a series of in vitro experiments were designed using human neuroblastoma SH-SY5Y cells, known by their tolerance to thermal stress. The application of two AMF cycles (30 minutes each) showed that the exogenous magnetic hyperthermia resulted in an 85% reduction of cell viability. This capacity of the TMFs of hyperthermia-mediated cell killing using a remote AMF opens new options for the treatment of small and superficial tumor lesions by means of remotely-triggered magnetic hyperthermia.

1 Introduction

The design of stimuli-responsive materials prepared through the incorporation of nanoparticles (NPs) into multilayer polymer films has gained increasing attention for the development of advanced functional materials employed as optical and humidity sensors,\textsuperscript{1,2} nanoelectroactuators,\textsuperscript{3} antibacterial\textsuperscript{4} or flame retardancy materials\textsuperscript{5} and flexible energy and electronic devices\textsuperscript{6,7} among other applications. Layer-by-layer (LbL) assembly, which uses complementary interactions between components to deposit materials one layer at a time, constitutes one of the most versatile methodologies for the production of stratified poly-electrolyte films and the incorporation of nanoparticles into these films.\textsuperscript{8,9} Specifically, magnetic nanoparticles (NPs) provide polymer films with the ability to respond to a magnetic field,\textsuperscript{10–12} and this magnetic responsiveness has been already used in biomedicine for controlled drug release,\textsuperscript{13} tissue engineering\textsuperscript{14,15} and magnetic hyperthermia (MHT) therapy.\textsuperscript{16}

The basis of MHT is related to the rise the temperature of target tissues or cells up to values \( \approx 43–46 \, ^\circ \text{C} \), triggering the apoptotic response of cells. The increase of the temperature is achieved by using magnetic nanoparticles used as heating agents under the action of an external AMF.\textsuperscript{17–20} The specific power absorption (SPA) is the power absorbed by unit mass of NPs, which is dissipated and heats the surrounding medium. There is a large number of works on NPs based on iron oxides, mostly \( \text{Fe}_3\text{O}_4 \) (magnetite) and \( \gamma\text{-Fe}_2\text{O}_3 \) (maghemite), reporting that the SPA values of these materials are large enough to heat macroscopic tumors. The generalized use of iron oxides is mainly due to the lower toxicity of this phases when compared to other magnetic heating materials such as Co, Ni and their oxides.\textsuperscript{21} In local hyperthermia, heat is applied to small and superficial tumor areas with the aim of reaching temperatures up to 42 \( ^\circ \text{C} \) for one hour within the cancer tumor to cause cell death. Polymer films with embedded gold nanoparticles have been successfully employed for tissue laser ablation when placed in contact with the tissue to be treated inducing local exogenous hyperthermia. While the photothermal therapy offers the possibility to control the energy dose delivered to the target area by both the intensity of the laser beam and the concentration of NPs, this technique is limited by the short penetration depth of the NIR wavelength into the human body \(( \approx 0.3 \text{ cm} \) in breast tissue) even at the known ‘optical window’ of water.\textsuperscript{22}

The exogenous heating strategy using magnetic fields that we report in this work can deliver energy density values to the target area that are lower than the typical values produced by photothermal therapy. However, as biological tissues essentially do not interact with ac magnetic fields of 100–500 kHz, the energy can be delivered essentially into any depth of the human body.
Clinical cases where local hyperthermia is a viable alternative include chest wall recurrences, superficial malignant melanoma lesions and head/neck tumors.\textsuperscript{23,24} In these cases, the application of the remote magnetic fields is achieved by adapted superficial applicators of different shapes and kinds wired to the power generators of radiofrequency generators, microwave, ultrasound or near infrared radiation (NIR).\textsuperscript{25-27} To the best of our knowledge, the potential employment of thermomagnetic films which could be more adapted to subcutaneous or deeper applications has not been proposed before.

In this study, we evaluate the thermal effect and show a proof of concept at the \textit{in vitro} level of the application in local magnetic hyperthermia treatment of thermomagnetic polymer films constituted of alginate, chitosan and a lab-made magnetic ferrofluid obtained through spray-assisted LbL from the corresponding aqueous polyelectrolyte solutions.\textsuperscript{28} Spray assisted LbL constitutes an attractive alternative to dipping or spin-coating LbL because it is much faster and easier to adapt at an industrial level.\textsuperscript{12} Human neuroblastoma SH-SY5Y cells were selected for proving the proposed application in local magnetic hyperthermia of the TMFs as a model of human malignant metastatic neuroblastoma which exhibits a notable resistance to hyperthermic stress.\textsuperscript{29}

\section*{2 Experimental section}

\subsection*{2.1 Materials}
Chitosan (Chi) of low molecular weight was supplied by Aldrich (448869, lot SLBG1673V). According to the fabricant, viscosity was 20–300 cps (1 wt\% in acetic acid, 25 °C, Brookfield). This chitosan was purified using the procedure described by Signini and Campa\-nha Filho.\textsuperscript{30} The deacetylation degree (DD) was determined by RMN\textsuperscript{-1}H at 70 °C using 2 wt\% CD\textsubscript{3}COOD/D\textsubscript{2}O as solvent and it was 81%.\textsuperscript{31} Molecular weight (M\textsubscript{w}) determined by capillary viscosimetry at 25 °C using as solvent acetic acid 0.3 M per sodium acetate 0.2 M and applying the equation of Mark–Houwink (k = 74 × 10\textsuperscript{-5} dl g\textsuperscript{-1} a = 0.76)\textsuperscript{32} was 67 000 Da.

Sodium alginate (Alg) was supplied by Sigma-Aldrich (A2158, lot 090M0092V). According to the fabricant, viscosity was 136 cps (2\% w/v in water at 25 °C). Molecular weight (M\textsubscript{w}) determined by capillary viscosimetry at 25 °C using as solvent sodium chloride 0.1 M and applying the equation of Mark–Houwink (k = 2 × 10\textsuperscript{-5} dl g\textsuperscript{-1} a = 1.0)\textsuperscript{33} was 166 000 Da.

Poly(ethyleneimine) (PEI) with a molecular weight (M\textsubscript{w}) of 25 000, acetic acid, were supplied by Aldrich and used as received. Sodium acetate anhydrous was supplied by Panreac and chloride acid by VWR.

Live/dead viability/cytotoxicity kit, for mammalian cells (Molecular Probes, ThermoFisher scientific, L3224). The kit contains calcein AM (4 mM in anhydrous DMSO) and ethidium homodimer-1 (2 mM in DMSO/H\textsubscript{2}O 1:4 (v/v)).

\subsection*{2.2 Preparation and characterization of lab-made ferrofluid}
A lab-made aqueous ferrofluid was synthesized by a coprecipitation method or iron salts carried out in a polymer aqueous solution. Briefly, 0.834 g of iron(II) sulfate heptahydrate (FeSO\textsubscript{4}\cdot7H\textsubscript{2}O) and 16.218 g of iron(III) chloride hexahydrate (FeCl\textsubscript{3}\cdot6H\textsubscript{2}O) were dissolved in 10 mL of Milli-Q water under N\textsubscript{2} atmosphere. Then, the mixture was added to a three-necked flask bubbled containing 40 mL of alginate (2.5 mg mL\textsuperscript{-1}) under N\textsubscript{2} atmosphere and mechanical stirring at 350 rpm using a teflon stirrer and heated at 80 °C for 1 hour. After that, this mixture was added drop wise under constant stirring to other three-necked flask bubbled containing 50 mL of ammonium hydroxide (NH\textsubscript{4}OH), taking place the color change of the mixture from yellow orange to black. The mixture was held for 2 hour at 80 °C under stirring and N\textsubscript{2} atmosphere. Afterwards, the solution was cooled in an ice bath and washed with Mill-Q water until neutral pH giving rise to an alginate based magnetite ferrofluid.\textsuperscript{34}

The determination of iron content of the ferrofluid was carried out through UV-Vis transmission spectrophotometry using the thiocyanate complexation reaction and measuring the absorbance of the iron–thiocyanate complex at 478 nm wavelength.

\begin{equation}
\text{Fe}^{3+} + 6\text{SCN}^{-} \rightarrow [\text{Fe(SCN)}]_{6}^{-}
\end{equation}

Ferrofluid was dissolved in HCl 6 M/HNO\textsubscript{3} (65\%) for 2 h. After that, potassium thiocyanate was added to the solution to form the iron–thiocyanate complex.

The ferrofluid is constituted by magnetite (Fe\textsubscript{3}O\textsubscript{4}) NPs dispersed in a sodium alginate solution at a concentration of 8 mg mL\textsuperscript{-1} as determined by UV-Vis spectrophotometry with an average size of 7.3 ± 1.1 nm as determined by transmission electron microscopy.\textsuperscript{34}

The heating capacity of the as prepared ferrofluid under alternating magnetic fields (AMF) was measured using a commercial magnetic field applicator (DM-100, nB Nanoscale Biomagnetics, Spain) at frequencies 250 < f < 820 kHz and field amplitudes 0 < H < 24 kA m\textsuperscript{-1}. Calorimetric measurements were carried out at thermal insulated conditions using a vacuum pump (10\textsuperscript{-7} mbar). A fibre-optic thermometer integrated into this equipment allowed to measure the temperature as a function of time.

In order to evaluate the heating mechanism of NPs immobilized in gelatin gels, 500 \textmu L of ferrofluid with a concentration of 8.0 mg mL\textsuperscript{-1} were precipitated at 14 500 rpm for 15 min, then the supernatant was removed and NPs were dispersed in 1 mL of 1% w/v or 2% w/v gelatin in a vortex for 1 min and kept at −4 °C for 1 hour to form the gel. After that, SPA was measured in the commercial equipment DM-100 (NanoScale Biomagnetics) at a frequency of 571 kHz and a magnetic field amplitude of 24 kA m\textsuperscript{-1}.

\subsection*{2.3 Preparation and characterization of thermomagnetic polymer films}
All films were built on glass slides (12 mm 2) previously cleaned with ethanol and rinsed extensively with water. As the glass substrate is negatively charged, a layer of PEI (1 mg mL\textsuperscript{-1}) was deposited as a first layer to get a homogenous substrate positively charged by dipping the substrate in a solution of PEI for 5 minutes and then in a solution of distilled

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water for 2 minutes. Substrates were dried to begin the experiment. Substrate was inclined 45° in regard to the vertical support to allow the drainage of the solution. The multilayer films were built by a spray assisted layer-by-layer (LBL) technique of aqueous solutions of alginate (2.5 mg mL⁻¹), chitosan (1 mg mL⁻¹) and a lab-made ferrofluid containing the NPs (8.0 mg mL⁻¹). The spray time was fixed at 5 s with a waiting time of 15 s between layers of chitosan and alginate and 30 s between NPs and Chitosan layers. The cycle was formed by a bilayer Alg/Chi followed by four bilayers NPs/Chi and repeated as many times as necessary until the desired number of layers was obtained. In order to achieve freestanding films, a polyvinyl alcohol (PVA) layer with a concentration of 100 mg mL⁻¹ was deposited over the films using a pipette and dried for 24 hours until become robust. The resulting multilayer film with the ending PVA layer was peeled from the glass substrate employing tweezers; after that, the resulting freestanding film was immersed in Milli-Q water and the PVA layer was removed by dissolution in water.

The determination of iron content of the (Alg/Chi)ₙ(NPs/Chi)ₘ films was carried out following the same procedure as that used for the ferrofluid.

2.4 Cell culture and adhesion experiments

Direct contact assays to test cell adhesion were carried out on (Alg/Chi)ₙ(NPs/Chi)ₘ films with different number of layers. Films were sterilized under UV light for 5 hours. Human neuroblastoma SH-SY5Y cells (ATCC CRL-2266) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 (1 : 1) with 15% fetal bovine serum, 10 IU mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and 2 mM L-glutamine. Cells were maintained at 37 °C in a saturated humidity atmosphere containing 95% air and 5% of CO₂. At confluence, cells were trypsinized and seeded on the films surface (1.5 × 10⁵ cells per film). Cells were cultured on (Alg/Chi)ₙ(NPs/Chi)ₘ films for 48 hours.

2.5 Dual beam (FIB-SEM) analysis

Dual beam FIB/SEM (Nova 200 NanoLab, FEI Company) was carried out on (Alg/Chi)ₙ(NPs/Chi)ₘ films in order to determine, on the one hand, the inner organization of the polymer films and, on the other hand, observe the adhesion of SH-SY5Y cells onto the surface of the polymer films. Cells adhered onto the films were fixed using 2% glutaraldehyde for 2 h at 4 °C and treated with 2.5% potassium ferrocyanate and 1% osmium tetroxide. Later, SH-SY5Y cells were dehydrated and coated with platinum for imaging using the FIB-SEM. SEM images were taken at 5 kV employing a PEG column, and a combined Ga-based 30 kV (10 pA) ion beam was used to cross-section single cells. These investigations were completed by energy-dispersive X-ray spectroscopy (EDX) for chemical analysis.

2.6 Determination of the thermomagnetic properties of the films

For the (Alg/Chi)ₙ(NPs/Chi)ₘ films, the heating capacity was measured in an open experimental setup, i.e., no thermal insulation. The magnetic field was applied using a commercial induction heating station (EasyHeat, Ambrell B.V., The Netherlands) working at f = 180 kHz and field amplitude of $H = 35$ kA m⁻¹. The samples were placed at the center of 11-turn solenoid, and thermalized at 37 °C by a water-circulating jacket, which has also the function of thermally detach the samples from the joule heating generated by the coil. The temperature was measured using a thermal camera (FLIR Systems, USA) in a zenithal position over the film. Before each experiment, the samples were placed inside the solenoid and allowed to thermalize at 37 °C for 5 min before the AMF was turned on (Fig. S1, ESIT). The heating time for each sample was 5 min (Fig. S2 shows representative results obtained for a (Alg/Chi)ₙ(NPs/Chi)ₘ film, ESIT).

2.7 In vitro hyperthermia treatment of SH-SY5Y cells

Two different experiments were performed to analyze the heating efficiency of (Alg/Chi)ₙ(NPs/Chi)ₘ films, in order to test as a suitable material for magnetic hyperthermia (MHT) applications. In the first experiment, 1.5 × 10⁵ human neuroblastoma cells (SH-SY5Y) were seeded and cultured on the film surface for 48 hours in an Ibidi dish at 37 °C in a saturated humidity atmosphere containing 95% air and 5% of CO₂. After that, the film was subjected to 1 cycle of MHT ($f = 180$ kHz, $H = 35$ kA m⁻¹, $t = 30$ min). In addition, several films were exposed to three cycles of MHT within five hours between them. Cells were maintained at 37 °C in a saturated humidity atmosphere 95% air and 5% CO₂ between each cycle.

A second experimental protocol consisted on placing a thermomagnetic film above 1.5 × 10⁵ human neuroblastoma cells (SH-SY5Y) cultured on an Ibidi dish for 48 h at 37 °C in a saturated humidity atmosphere containing 95% air and 5% of CO₂. After that, the Ibidi dish was subjected to two cycles of MHT ($f = 180$ kHz, $H = 35$ kA m⁻¹, $t = 30$ min, waiting time between cycles = 5 h).

Viability of cells cultured on (Alg/Chi)ₙ(NPs/Chi)ₘ control films was determined using the live/dead viability/cytotoxicity kit. Viability of cells subjected to MHT was measured, as described above, 12 hours after the application of the last MHT cycle in the first experimental protocol and after 3 hours in the second experimental protocol. The kit contains calcein AM (4 mM in anhydrous DMSO) and ethidium homodimer-1 (2 mM in DMSO/H₂O 1 : 4 (v/v)) which allows staining live cell in green and dead cells in red, respectively. Cells were washed with 1 mL of PBS, after that, 1 mL of the stain kit was added and kept for 30 minutes at room temperature in darkness. The stained cells were visualized using a Leica SP2 AOBS confocal scanning microscope. Images were collected using the microscope in sequential mode using a 20× (lens specification, HCPL FLUOTAR NA 0.50; Leica), a line average of 16 and a format of 1024 × 1024 pixels. The confocal pinhole was 1 Airy unit. The cell viability assay was carried out for three different samples for each measurement. The results shown in this paper correspond to the average value of percentage of cell viability obtained by comparison of the live cell area of every sample in regard to the live cell area of the control. The software ImageJ was used to calculate the area covered by live cells.
3 Results and discussion

3.1 Preparation and morphological characterization of the multilayer iron oxide-polymer films

Polymer/NPs films can be assembled by alternately spraying a substrate, coated with a branched polyethylenimine (PEI) primer layer, with solutions of alginate (2.5 mg mL\(^{-1}\)), chitosan (1 mg mL\(^{-1}\)) and a lab-made aqueous ferrofluid. Alginate and chitosan are known to establish electrostatic interactions between the protonated amines of chitosan and carboxylate groups of alginate.\(^{35}\) The repeating structural unity was formed by a bilayer Alg/Chi followed by four bilayers NPs/Chi as illustrated in the Fig. 1a. Samples were denoted as (Alg/Chi)\(_n\)(NPs/Chi)\(_m\) where \(n\) stands for the number of Alg/Chi bilayers (\(n = 2, 4, 8, 12, 20\) and 40) and \(m\) for the number of NPs/Chi bilayers (\(m = 8, 16, 32, 48, 80\) and 160).

The incorporation of NPs within the multilayer structure of films was assessed visually (Fig. 1b), as can be observed, films become darker as the number of NPs layers increases. The magnetite content (Fe\(_3\)O\(_4\)) incorporated within the multilayer films was determined by UV-Vis transmission spectrophotometry and plotted in Fig. 1c as a function of the number of NPs layers. The magnetite content increases linearly with the number of NPs layers, that could be fitted by a \(f(\text{Fe}_3\text{O}_4) = -0.44 + 0.052N\), where \(N\) is the number of NPs layers. The linear trend observed up to the maximum number of NPs layers deposited suggests that the final Fe\(_3\)O\(_4\) concentration per unit area could be even increased, although we observed the formation of cracks on the surface of films with \(N > 200\) so that it was only possible to form robust films until \(N = 200\). However, as discussed below, the deposition of the NPs layers during the process of build-up of multilayer polymer films was enough to produce a large (i.e., \(T > 10^\circ\)C) temperature increase during the magnetic hyperthermia experiments.

The inner structural organization of (Alg/Chi)\(_n\)(NPs/Chi)\(_m\) multilayer films was characterized through dual-beam (FIB-SEM) images. Representative results corresponding to the cross section of sample (Alg/Chi)\(_n\)(NPs/Chi)\(_{12}\) are shown in Fig. 2a. A magnification of the SEM image shown in Fig. 2b allows to observe some degree of nanostructuralization in layers of the sample. The thickness of a film with 32 NPs layers, determined by SEM, is \(~1\) \(\mu\)m (Fig. 2b). Considering a linear growth due to the fact that the iron content follows a linear tendency as the number of NPs layers increases (Fig. 1c), the thickness of a film with 160 NPs layers could be estimated as \(~5\) \(\mu\)m. An EDX analysis of these cross-sectioned samples revealed the presence of NPs layers (Fig. 2c and d–f) as shown from elementary Fe mapping of the material. The carbon is due to the polysaccharides, alginate and chitosan.

3.2 Determination of the heating efficiency of the TMFs

The heating efficiency of single-domain NPs is related to two main mechanisms of magnetic relaxation, i.e., Brown and Néel relaxation. The former is associated to the relaxation by

Fig. 1  (a) Schematic representation of the spray assisted layer-by-layer procedure and the repeating structural unit of the multilayer films. Notice that NPs are coated by alginate (pink colour) due to the preparation procedure of the lab made ferrofluid. (b) Image of films assembled on glass substrates with different number of NPs from 0 to 160 as indicated in the figure. (c) Determination of Fe\(_3\)O\(_4\) content as a function of the number of NPs layers deposited. Dashed line represents the linear fit of the data.
physical rotation of the NPs against viscosity forces, whereas the latter corresponds to the rotation of the magnetic moment in regard to the crystal lattice. Therefore Brown relaxation is stalled when NPs are incorporated into the LbL films, making relevant to determine how the heating efficiency is changed from the as prepared colloids after the NPs are immobilized. To evaluate this, the NPs of the original colloid were immobilized in gelatin gel, and the resulting SPA values compared to the values from the NPs in the as prepared colloids.

The SPA of the as prepared colloids was quite high compared to most reports from the literature, i.e. SPA = 868.4 ± 1.9 W g⁻¹ (see Fig. 3)⁴⁶,⁴⁷ although the actual efficiency is determined by the SPA of blocked NPs as discussed above.⁴⁸ As expected, the SPA decreased after immobilization in gelatin and this decrease is higher as gelatin concentration increases. The exposition of magnetic NPs to a radio-frequency magnetic field gives rise to a single domain particle that aligns its magnetic moment M to the field H to decrease the interaction free energy. There are two dissipation mechanisms, Brownian relaxation and Néel relaxation, which influence the magnetization of ferrofluids when they are exposed to external time varying magnetic fields. Brownian relaxation is due to the physical rotation of the nanoparticles against viscous torques from the surrounding media, i.e., carrier liquid, films, etc., and Néel relaxation is caused by the rotation of the magnetic moment inside the magnetic core.⁴⁹,⁵⁰

Due to the fact that both mechanisms take place simultaneously in the dissipation mechanism of the ferrofluid, the immobilization of NPs in gelatin tends to block the physical rotation of NPs as gelatin concentration increases and, subsequently, the Brownian contribution to the SPA value decreases being the Néel relaxation the main dissipation mechanism. Therefore, in our system it is expected that the same physical process will take place when NPs are encapsulated into the multilayer films based on chitosan and alginate as it will be discussed below.

To assess the efficiency of the thermomagnetic films produced, a series of (Alg/Chi)_n(NPs/Chi)_m films with different number of NPs layers were heated under a fixed AMF amplitude and frequency. The temperature increase, ∆T, measured from the basal value T₀ = 37 °C, is plotted as a function of time (Fig. 4a) and as a function of the NPs layers (Fig. 4b) for the different samples. The heating of the films, from the basal value T₀ = 37 °C, as a function of the time by action of the AMF was followed for 5 minutes for the different samples (Fig. 4a). As can be observed, temperature increases during the first two minutes, then a plateau is reached. It can be seen from Fig. 4b that ∆T increases linearly with the number of NPs layers (i.e., films with 80 or 160 NPs layers yield ∆T of 6 and 12 °C, respectively). Notice that
the magnetic heating of the ferrofluid with a magnetite concentration of 8.0 mg mL$^{-1}$ gave rise to a temperature increase of 25 °C in 1.5 min (Fig. S3, ESI†) and nanocomposite films with 80 and 160 NPs layers give rise to a temperature increase of 5.8 and 11.2 °C in 1.5 min, respectively (Fig. 4a). The immobilization of NPs into a multilayer Alg/Chi film through spray LbL produces a similar effect to this observed for the immobilization of NPs into a gel-like material decreasing the temperature increase (ΔT) in regard to the ferrofluid.

### 3.3 **In vitro cell studies**

As (Alg/Chi)$_n$(NPs/Chi)$_m$ films are intended to be employed as adhesive pads for exogenous magnetic hyperthermia treatment, the first step in order to optimize the proposed application was to ascertain the cell adhesion onto the surface of the thermomagnetic films. Fig. 5a shows a general view of neuroblastoma cells cultured for 24 h on a (Alg/Chi)$_{20}$(NPs/Chi)$_{80}$ film where cell adhesion and proliferation can be observed along the film surface. A magnification of the SEM image (Fig. 5b) allows observing an individual cell adhered onto the film surface.

Further information on cell adhesion can be obtained from the cross section of the film. The cell adhered completely to the film surface as can be observed in a lamella cross section of the cell shown (Fig. 5c). The analysis of the EDX spectrum corresponding to this area (Fig. 5d) with the different elements, carbon in green (Fig. 5e), iron in red (Fig. 5f), oxygen in grey (Fig. 5g) and silicon in blue (Fig. 5h) allows to clearly differentiate the iron component embedded on the film (red color) from the nucleus of the cell (black color) marked by the arrow. Note that the presence of silicon is due to the fact that the film is supported on a glass substrate. The composition of the film can be quantitatively determined from the EDX spectrum (Fig. 5i) where the major component is iron (64.67%), followed by O (28.69%) and C (6.64%).

The effect of magnetic hyperthermia (MHT) on the cell viability was evaluated using neuroblastoma cells cultured on films with 80 and 160 NPs layers and subjected to 1 cycle of MHT ($H = 35.1$ kA m$^{-1}$ and $f = 180$ kHz; 30 min) and 3 cycles of MHT giving a waiting time of 5 h between cycles. The results were compared to those obtained for neuroblastoma cells cultured on a control film not subjected to MHT treatment. A schematic representation of the experimental procedure is shown in Fig. 6a.

Cell adhesion on the surface of biomaterials is influenced by different factors such as the substrate topography, rigidity, anisotropy, surface charge, and wettability. Taken into account this, the cellular response to specific surfaces constitutes a complex phenomenon that includes a wide range of physical external stimuli generated at the interface between the interface between cells and the biomaterial surface. The discussion of the mechanism of adhesion of SH-SY5Y cells on the multilayer films under study goes far beyond the scope of this paper; however some qualitative results can be extracted from the results shown in control experiments depicted in Fig. 6b and c. Films with 80 NPs layers and not subjected to MHT present a lower number of live cells (stained in green) adhered to the films that that observed for cells cultured on films with 160 NPs (Fig. 6c). This is in general agreement with the results found in literature regarding cell adhesion on multilayer films that show that, generally speaking, cells tend to adhere more on films which have a higher number of layers. Other studies have also shown that the incorporation of nanoparticles into polymer films improved cell adhesion due to the increase in surface roughness. In particular, human neuroblastoma cell line (SH-SY5Y) presents a remarkable response to surface nanotopography, and a surprising sensitivity to variations of few nanometers. For multilayer films based on Alg and Chi with NPs layers, the elastic moduli determined through PeakForce quantitative nanomechanical mapping atomic force microscopy (PF-QNM AFM) increased with the number of NPs layers, being higher than the corresponding to multilayer films of Alg and Chi without NPs. For example, in the case of multilayer films with $N = 40$, the elastic modulus for a film with NPs was 26.2 ± 3.9 GPa whereas the elastic modulus for the same film without NPs was 9.2 ± 1.6 GPa. Therefore, for the samples under study here, the increase of cell adhesion observed with the number of...
NPs layers could be attributed to a combination of both factors, a higher surface roughness and a higher stiffness of films as number of NPs layers increases.

For cells cultured onto films with 80 NPs layers (Fig. 6b), the application of one and three cycles of MHT does not significantly change the area covered by live cells in regard to the control experiment. In contrast, Fig. 6c shows that the application of one cycle of MHT on cells cultured on films with 160 NPs layers results in a decrease of area covered by live cell population in regard to the control film, being this decrease much more noticeable after the application of 3 cycles of MHT. It is worth to note that the percentage of dead cells stained in red (ethidium homodimer-1 in Fig. 6b and c) does not correspond to the total number of dead cells due to the fact that cell death results in the loss of cell adhesion and hence detachment from the film.

As can be observed in Fig. 6d, the cell viability data (%) obtained from the quantification of the area covered by live cells stained in green on films with 80 NPs layers demonstrates that the application of MHT results in negligible cytotoxic effects after the application of one (96% viable cells) or three MHT cycles (94% viable cells). However, the cell viability data (%) corresponding to neuroblastoma cells cultured on films with 160 NPs layers decreases to 69% in regard to the control experimental by the application of one cycle of MHT and to 21% by the application of three cycles of MHT. At this point, it is important to take into account the final temperature reached by the TMFs by the application of AMF which is related to the number of NPs layers as shown in Fig. 4a. TMFs with 80 NPs layers reach a final temperature of 43 °C, which under the experimental protocol employed, is not high enough to induce a cytotoxic effect on neuroblastoma cells whereas the temperature reached by TMFs with 160 NPs layers increases to 49 °C which induces a decrease of cell viability to 69% in regard to the control experiment. These results demonstrate the importance of controlling the concentration of NPs and hence, the temperature generated during the magnetic field application in order to produce a measurable magnetic hyperthermia effect. The temperature range for activating apoptotic cell mechanisms depends on the cellular type involved, but it can be generalized that above 43–45 °C different metabolic pathways are activated to reverse the effects of the temperature.18,46 For our experiments, the temperature gradient was expected to be large due to the bidimensional nature of the samples (and thus large surfaces for exchanging heat).

It can be observed from Fig. 6 that the films with 80 magnetic layers did not produce enough thermal effect to affect the cell viability even after three application cycles. However, for those films with 160 magnetic layers the application of several cycles increased the cell mortality through MHT. This is consistent with previous studies showing an increase in cell death in cell cultures subjected to several expositions to AMF.47,48 The stability of our films remained essentially unchanged after the magnetic hyperthermia experiments. Indeed, we would expect that excessive heating could result in damage of the polymers. However it is known that chitosan/alginate multilayers retain their integrity up to 200 °C,49 which is much higher than target temperatures in any clinical application.
4 Conclusions

We have successfully prepared thermomagnetic polymeric films by alternate deposition of chitosan and alginate layers and magnetic nanoparticles using a spray assisted layer-by-layer assembly. The layered structure, together with the ordered distribution and intercalation of NPs between layers, provided the expected mechanical stability and thermal responsiveness. Indeed, the concentration of NPs (up to \( \approx 65\% \) in mass) into the multilayer structure of these films was enough to produce a temperature increase of up to 12 °C above the control samples under external AMF. The resulting polymeric films showed good adhesion properties for neuroblastoma cells cultured on the film surface. By remotely heating the films using AMFs we were able to induce a reduction in cell viability down to 69% and 21% of the original population after one and three heating cycles, respectively. The results in experimental conditions mimicking in vivo applications yielded a reduction of 85% of the cell viability only within the contact area with the film after two cycles of MHT.

Conflicts of interest

There are no conflicts to declare.

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Notes and references
