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One-pot synthesis of magnesium nanoparticles embedded in chitosan microparticles matrix: a highly biocompatible tool for in vivo cancer treatment.

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Abstract. A novel highly biocompatible nanosystem made up of chitosan matrix and filled with magnesium nanoparticles was synthesized with a simple and one-pot strategy, and tested as promising, well-tolared tool for photothermal therapy. Moreover, in vivo Proof of Concept on hepatocarcinoma-bearing mice is presented.

Introduction

There is an enormous expectation surrounding the field of nanomedicine, the application of nanotechnology to healthcare.[1] Undoubtedly nanomaterials show new appealing therapeutic features whereas common drugs fail[2] but the eco-friendly production of nanomaterials is still scarcely investigated and need more findings. Moreover, nanotoxicity and health hazards on humans are concerns still not deeply engaged by the scientific community. Therefore there is urgency for nanomaterials to take up these burdens allowing novel green strategies toward novel materials with enhanced biocompatibility using safe reagents.[3]

Photothermal ablation therapy, which exploits localized heat increase of a few degrees to kill cancer cells, has appeared recently as a non-invasive, highly efficient and well-tolerated therapy against various cancer types.[4] New agents, which are able to generate therapeutic hyperthermia when irradiated are needed and must have precise biocompatibility in order to avoid damage to healthy tissues and prevent system toxicity-related events.

Safe metals in the form of nano- (NPs) and micro-particles (MPs) can be able to fulfil this task. Among them gold has been the mostly investigated as photothermal agent:[5] some disadvantages such as its accumulation in body and the need to formulate it with toxic surfactants are enough to cast doubts whether its use should be encouraged.[6]

The introduction of safer metals must be considered a priority in nanomedicine and recently there have been an increasing number of studies concerning magnesium as safe biomaterials.[7]

Magnesium is among the four most abundant metals in body, and covers fundamental roles in human biology. Safety of magnesium-based particles rely thus in the adsorption by the body of magnesium ions instead of excretion.[8]

The major drawbacks that have strongly limited the diffusion of magnesium particles till now are the high reduction potential of magnesium cations, which makes NPs or MPs synthesis challenging, and the tendency to re-oxidation once the NPs are formed, which can only be avoided with a highly efficient surface coating and stabilization.

We had already recently reported the synthesis of magnesium nanoparticles with a salt reduction methodology, their coating with organic ligand and their entrapment into polymeric micelles via oil-in-water technique.[9] Even if the so-obtained nanoparticles where stabile in water against oxidation, still the process required several steps and made use of synthetic polymer for the coating.

Chitosan is obtained as waste material by shrimps and other crustacean shells. It is a non-toxic, biodegradable and biocompatible natural sugar biopolymer with the advantages of being able to easily form nano- and micro-particles.[10] Considering its excellent versatility chitosan is being used in the fabrication of hybrid nanocomposite materials for biomedical applications including those based on photothermal activation.[11]

Hepatocellular carcinoma (HCC), the fifth most common cancer in men and seventh in women worldwide, accounts for the third major cause of cancer related deaths.[12] With more than 600 000 new cases of HCC yearly, HCC related death in US men is increasing with 2% rate.

Herein, we report the easy, one-step and one-pot synthesis of chitosan- microparticles containing very small magnesium NPs (Chit-Mg MPs): the proposed synthetic approach is fast, easy to be performed and leads to the obtainment of a powder, which is stable for months, easy to redispersed in water and

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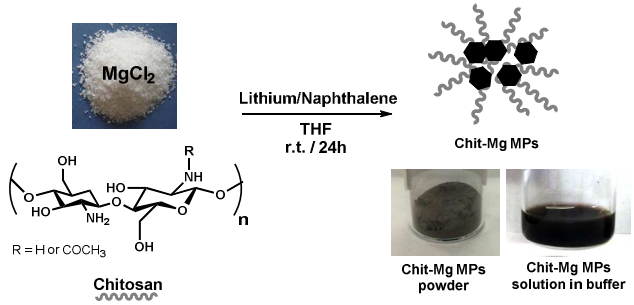
made only of safe and natural materials. Moreover, the clear demonstration of its use as heater for medical applications together with the exploitation for photothermal therapy of hepatocarcinoma in mice is also reported. The development of an easy procedure, the use of a more natural, biocompatible stabilizing and coating agent on a safer metal as the Mg would provide a larger widespread of this promising material.

Results and discussion

Synthesis

In order to obtain this system we slightly modify our previous methodology[9] by introducing chitosan-glutamate at the beginning of the process, simultaneously with the other reagents, thus having the coating agents present during the magnesium nanocrystals formation (**Scheme 1**). Briefly, $MgCl_2$ was reduced in tetrahydrofuran (THF) with metallic lithium and exploiting naphthalene as electron carrier; chitosan-glutamate was added to this solution without necessity to prior dissolution. Indeed chitosan presents no reducible sites by lithium-naphthalene complex, having only amino and hydroxyl groups in its backbone, thus allowing its survival in the strong-reducing reaction environment. Moreover both amino and hydroxyl groups have strong affinity for the magnesium nanocrystals surface, thus chitosan may form stable linkage onto the surface of magnesium particles stabilizing them either against aggregation and oxidation.



Scheme 1. Representative scheme of Chit-Mg MPs synthesis and photos of both powder and MPs re-dispersed in acetate buffer.

The best reaction conditions were evaluated. Particularly, chitosan-magnesium ratio was changed in order to identify the most suitable one. Three different syntheses with chitosan-$MgCl_2$ (w/w) ratio of 1-1, 2-1 and 5-1 were attempted and the resulting particles tested against oxidation in water. The results indicated that 1-1 ratio is not enough for a complete and efficient embedding of the magnesium particles since they quickly react with- and dissolved in water (hydrogen bubbles development and disappearance of the particles), forming the soluble species $Mg(OH)_2$. Both 2-1 and 5-1 ratios are suitable for the formation of stable microparticles that are dispersible in water but do not react with it. For this reason a 2-1 ratio was selected.

After 24 hours it is possible to recover the so-formed Chit-Mg MPs by centrifugation: a good purification is required in order to remove all the reaction by-products and in particular naphthalene and LiOH, deriving from the reducing agent. For this reason Chit-Mg MPs were washed three times with THF and centrifuged before drying under vacuum. Finally, they can be re-dispersed in acetate buffer (pH 4.6) or slightly acidic water (pH 5) without visible agglomeration. Notably the particles remained stable as dry powder for more than 10 months, while they were stable in solution for at least one month.

Dynamic light scattering (DLS) of Chit-Mg MPs revealed particles with hydrodynamic diameter of 1.15 ± 0.07 µm (**Figure S1**) and a positive surface charge of +38 mV, due to the presence of several protonated amino groups in the chitosan chains. In order to investigate size and morphology of the magnesium nanoparticles entrapped into the chitosan matrix Transmission Electron Microscopy (TEM) was performed (**Figure 1A-B**): images showed polygonal-shaped nanoparticles with diameter ranging between 5 and 10 nm. Indeed nanoparticles obtained with this synthetic methodology are smaller than the ones previously reported by us, a fact that can be attributed to the presence of a capping agent placed directly into the reaction mixture, allowing for an immediate stabilization of the particles and avoiding uncontrolled growth. Anyway, the DLS findings were in good agreement with scanning electron microscopy (SEM) images, which showed well homogeneous particles with approximately round shape and size slightly smaller than one µm (**Figure 1C-D**): the difference between DLS and SEM findings are to be considered the normalcy as DLS can only reveal hydrodynamic diameter, while SEM the effectively size. Energy Dispersive X-ray (EDX) analysis confirmed the presence of magnesium atoms, as well as of the chitosan organic matrix (**Figure S2**), which is indicated by the presence of oxygen and nitrogen atoms, in addition no residual lithium was found after purification of the MPs. Moreover, X-ray diffraction (XRD) analysis showed crystalline nanoparticles and the typical reduced-magnesium pattern (**Figure S3**).

A solution prepared by dissolving 10 mg of powder in 1 mL of buffer showed a concentration in magnesium equal to 589 mg/L (24.2 mM). Thermogravimetric analysis (TGA) was also recorded (**Figure S4**): firstly the degradation of the organic layer was observed between 150 and 350 °C; interesting when the gas flow was changed from nitrogen to air at 600 °C an immediate and intense weight gaining was observed, which can be attributable to the oxidation of the magnesium core, now uncoated due to the degradation of chitosan matrix, and thus prone to oxygen attack.

FTIR analysis (**Figure S5**) confirmed the presence of the organic matrix onto the surface of the NPs by showing the characteristic signal of chitosan: glycosidic linkage (–C–O–C–) at 1064 cm^{-1}, amines and protonated amines at 1510-1580 cm^{-1} and at 2800-2900 cm^{-1}, and intermolecular bonded –OH as broad band between 2800 and 3000 cm^{-1}.

To further characterize the material ζ potential at different pH (**Figure S6**) was checked: the graph suggested a good overall

stability from acidic environment to almost pH 8, with an isoelectric point at pH 8.4, as expected for chitosan, due to the presence of residual amino groups.

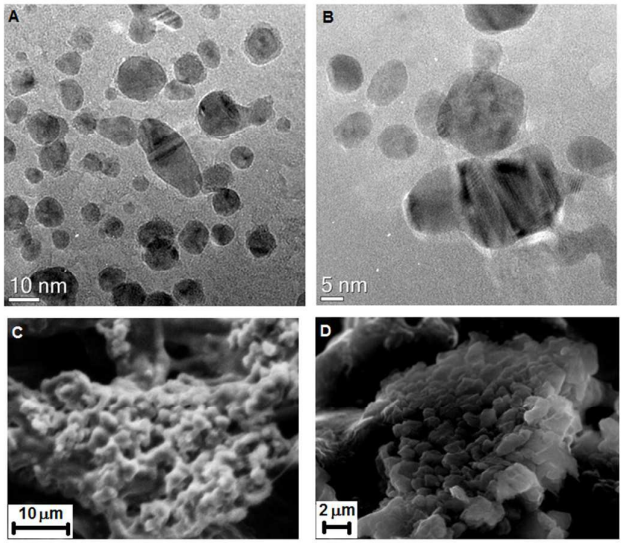


Figure 1: A-B) TEM images and C-D) SEM images of Chit-Mg MPs after vacuum dryer. Lower magnification (left side) and higher magnification (right side).

Photothermal behaviour

In a previous paper we provided the proof of concept that a NIR laser illumination can be used to trigger a temperature rise from an aqueous dispersion of magnesium particles.[9] It is worth noting that the use of a NIR light is highly preferable with a view to biomedical applications inside the body due to its maximum penetration into biological tissues.[13]

Here we extended the investigation to the as-synthesised Chit-Mg MPs with the aim of proving their photothermal behavior at different concentration values (**Figure 2a**). A consistent photothermal response was observed once the particles were illuminated using a 810 nm diode laser operating in continuous wave (cw) mode at 13 W cm^{-2}. Starting from physiological temperature, we observed a uniform and characteristic temperature profile including a temperature rise as soon as laser light is turned on and a fast cooling phase after switching off the laser. Particularly, after 0.5 – 1 minute of irradiation, the temperature enhancement was enough to reach temperature values of interest for photothermal therapy (i.e. ~40 to 50 °C) despite of the concentration considered (**Figure 2b**).[14] An exemplary map of temperature registered after 3 minutes of irradiation at 13 W cm^{-2} is displayed in **Figure 2b** and highlights the confinement of the photothermal effect within the illuminated laser spot Immediate outcomes include an effective and well-localized photothermal response that can be obtained with particle concentrations typically employed within the protocols of photothermal therapies.[15]

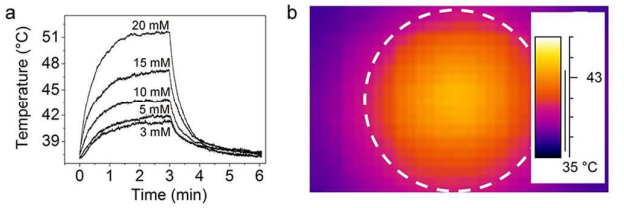


Figure 2: (a) Temperature profiles obtained by illuminating Chit-Mg MPs solutions at different concentration with a laser intensity of 13 W cm^{-2} and a t_{irr} = 3 min **(b)** Exemplary map of temperature registered after 3 min of irradiation of a 10 mM Chit-Mg MPs solution at 13 W cm^{-2}. The illuminated area is displayed by a dashed line.

In vivo cancer therapy

Biocompatibility of Mg@Chit MPs were firstly assessed by cell viability studies on hepa1-6 cell line (**Figure S7**). Cells incubated with the particles showed no mortality both at 48 or 72 hours in a magnesium concentration range of 0-100 µg/mL, which are standard time points and concentrations values when evaluating the toxicity of a novel nanosystem.[16]

In vivo photothermal therapy was then performed on xenograft hepa1-6 tumor bearing mice. Tumor cells where injected in the mice flank and when tumor grew to about 1 cm^2, the animals were treated with Chit-Mg MPs at 5 mM as well as with the same dosing of saline as per control. After 12 hours the tumor was then exposed to near-infrared (NIR) laser light. The power density was first optimized by a pilot study, and 1 or 3 W/cm^2 was chosen as the working scale for NIR light exposure. Then, the animals with the treatments of Chit-Mg MPs and saline were illuminated on the tumor sites for 0.5 or 2 minutes (**Figure S7**). No animals showed sign of suffering or died after the injection of the MPs. Clearly, in the area subjected to photothermal therapy the most tumor cells were destroyed.

We note that lower laser intensities were required in vivo, as compared to those employed for effective irradiation of nanoparticle solutions. This is explained by an accumulation of the particles at the tumor site and major heat confinement, according with previous findings.[17]

Three days after NIR exposure, the tissues were harvested for histological analysis. The results (**Figure 3**) clearly showed an extensive damage to tumor tissue after only 2 minutes of laser irradiation, while no damage was recorded when the tumor was treated with laser and saline alone.

In the normal control, without tumor cells inoculation, the normal histology showed layers structure, epidermis, dermis and hypodermis, and muscle while in the case of tumor treated with saline and laser did not affect the tumor mass. When Chit-Mg MPs was administrated with intratumoral injection to the tumor, laser exposure for 0.5 minute and 3 watt/cm^2 cause tumor cell death but it did not affect the benign muscle. When laser exposure was increased up to 2 minutes, most tumor cells were ablated and again the benign muscle was unaffected.

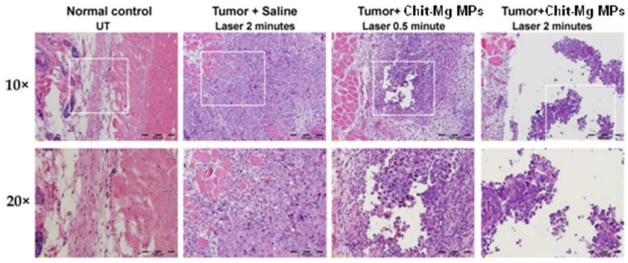


Figure 3. Histological changes of tumor with Chit-Mg MPs and laser exposure.

Conclusions

We reported a novel synthesis of a safe and highly biocompatible nanomedicine made of Mg NPs embedded into chitosan-based microparticles.
In addition, we demonstrated that laser irradiation of Chit-Mg MPs solutions generates temperature gradients of several degrees at concentration values usually considered for photothermal therapy. Proof of concept was also reported on HCC-bearing mice which shown a potential clinical impact of this safe and green nanomaterial for future clinical translations.

Experimental section

Material and methods.
All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Chemicals were of analytical grade and were used without further purification. All aqueous solutions were prepared with deionized water obtained using an ultrafiltration system (Milli-Q, Millipore) with a measured resistivity above 18 MΩ/cm. SEM images were acquired with a SEM Zeiss mod. EVO 50' EP. Energy dispersive X-ray spectroscopy (EDX) was performed with INCA x-sight, OXFORD instruments. Transmission Electron Microscopy (TEM) was conducted on a Jeol JEM 2010 at 200 keV. Samples for TEM analyses were prepared by spreading a small drop of the nanoparticle dispersion on amorphous carbon-coated copper grids (Formvar carbon 400 mesh grids) followed by air-drying. Fourier transform infrared (FTIR) spectra were recorded on a Perkin-Elmer Spectrum 2000. DLS measurements were performed on a Malvern Zetasizernano-S working with a 532 nm laser beam. ζ potential measurements were conducted in DTS1060C-Clear disposable zeta cells at 25°C. Magnesium concentrations of the samples were measured by atomic absorption spectroscopy (AAS) in a SpectraAA 100 Varian instrument. The crystalline structure of the samples was identified from X-Ray diffraction (XRD) patterns recorded in the 2θ range 10–70° with a scan step of 0.05°(2θ) for 5s on a Philips X'pert pro diffractometer (Cu K α radiation). Thermogravimetric analysis (TGA) was performed using a TA Instruments TGA-SDT 2960 on sample sizes from 3 to 5 mg, and the mass was recorded as a function of temperature.

Synthesis of Chit-Mg MPs
In a typical procedure $MgCl_2$ (142 mg, 1.5 mmol), naphthalene (192 mg, 1.5 mmol) and chitosan-glutammate (284 mg) were dispersed under vigorous stirring in anhydrous THF (10 mL) under argon, then lithium (37 mg, 5.4 mmol) was added: the reaction started after 20 minutes highlighted by the color change from white to black. After 24 hours the microparticles were centrifuged (6000 rpm) and purified by washing 3 times with 20 mL of anhydrous THF in order to remove all the by-products. The so-obtained Chit-Mg MPs were recovered as a black powder, dried under vacuum and store at +4 °C. For all the analysis 10 mg of powder were redispersed in 1 mL of acetate buffer (pH 4.73) or in acidic water (pH 5) using an ultrasound bath for 5-15 minutes.

Photothermal activity of Chit-Mg MPs. In a preferred configuration, 50 µL of Chit-Mg MPs solution were poured into a 0.5 cm^2 polystyrene well. A heating stage was used to keep the temperature constant at 37 °C. A 0.15 cm^2 area of the solution was illuminated by a 600 µm core optical fiber coupled to a 810 nm AlGaAs diode laser (Mod. WELD800, El.En. S.p.A.) and kept at a distance of ~2 cm from the sample surface. The temperature was measured by an infrared thermocamera (Thermovision A20 FLIR PD2A). Maximum temperature values reached within the irradiated area were collected during the laser illumination.

Xenograft tumor model
To establish a xenograft HCC model, male C57L/J mice (Jackson Laboratory, Bar Harbor, ME) at 8 weeks old and a mouse hepatoma cell line, hepa1-6, were used. C57L/J mouse is syngeneic with BW7756 mouse, while the hepa1-6 cell line is derivative of the BW7756 mouse hepatoma and shows reliable tumor growth in the syngeneic host C57L/J mouse. The hepa1-6 cells were inoculated at 10^6 cells into the right flank of C57L/J mouse. The tumor growth was observed daily. Animal procedures were approved by the Institutional Animal Care and Use Committee of University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care.

Chit-Mg MPs administration and NIR light exposure.
When the tumor growth reached 1 cm^2, the animals were injected intratumorally with Chit-Mg MPs solution in saline or with the same amount of saline solution for the control group. After 12 hours mice were anesthetized and exposed to a 808 nm light produced by a Diode Pumped Solid State laser (DPSSL) system . Three days after treatment animal were sacrificed and tumor tissues collected for histopathology examination.

Histopathology
The entire tumor tissues after laser exposure along with control tissues were collected and fixed in 10% buffered formalin for histological examination. The formalin-fixed tumor tissues were embedded in paraffin. Serial sections of 5-µm were mounted onto glass slides for histopathological

analysis. Hematoxylin and eosin (H&E)-stained slides were obtained for each sample for the histological study.e

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One-pot synthesis of magnesium nanoparticles embedded in chitosan microparticles: a highly biocompatible tool for in vivo cancer treatment

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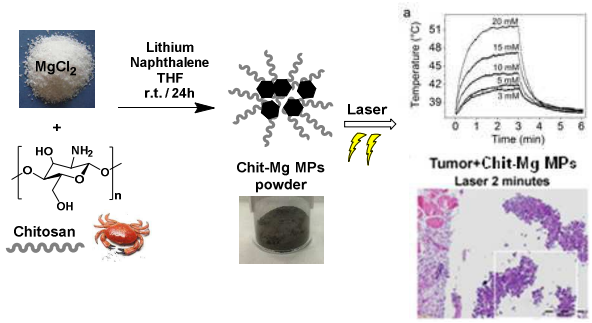

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