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Simultaneous encapsulation of magnetic nanoparticles and zinc phthalocyanine in poly(methyl methacrylate) nanoparticles by miniemulsion polymerization and *in vitro* studies

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ABSTRACT

The aim of this work was the simultaneous encapsulation of magnetic nanoparticles (MNPs) and zinc(II) phthalocyanine (ZnPc) in poly(methyl methacrylate) (PMMA) (MNPsZnPc-PMMA) nanoparticles (NPs) by miniemulsion polymerization and to evaluate the photobiological activity and/or hyperthermia (HPT) against human glioblastoma cells (U87MG). MNPsZnPc-PMMA NPs presented an average diameter of 104 ± 2.5 nm with a polydispersity index (PdI) of 0.14 ± 0.03 and negative surface charge -47 ± 2.2 mV (pH 7.4 ± 0.1). The encapsulation efficiency (EE%) of ZnPc was $85.7\% \pm 1.30$. The release of ZnPc from PMMA NPs was slow and sustained without the presence of burst effect, indicating a homogeneous distribution of the drug in the polymeric matrix. In the biological assay, MNPsZnPc-PMMA NPs showed considerable cytotoxic effect on U87MG cells only after activation with visible light at 675 nm (photodynamic therapy, PDT) or after application of an alternating magnetic field. The simultaneous encapsulation of MNPs and ZnPc in a drug delivery system with sustained release can be a new alternative for cancer treatment leading to significant tumor regression after minimum doses of heat dissipation and light.
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1. Introduction

The development of polymeric nanoparticles (NPs) loaded with hydrophilic drugs has been attracting considerable interest in recent years for cancer treatment through targeted administration to specific tissues or cells, with controlled drug delivery [1,2]. Miniemulsion polymerization allows the production of polymeric NPs with unique characteristics of great interest for biomedical applications [3]. The miniemulsion polymerization technique may be an advantageous encapsulation method, including the ability to control the particle size, directly dispersing the hydrophobic inorganic particles in the monomer phase with the aid of a stabilizer, the ability to nucleate all the droplets containing inorganic particles, as well as presenting a faster polymerization and, depending on the formulation, resulting in biocompatible NP dispersions [4–6]. Poly(methyl methacrylate) (PMMA) is a biocompatible and non-biodegradable polymer prepared by miniemulsion polymerization that has been used for drug delivery [3,6]. Since it is non-biodegradable, it can increase the circulation time in bloodstream, reaching therapeutic index and reducing side effects of the encapsulated drugs [7]. NPs are promising delivery systems for use in photodynamic therapy (PDT) and hyperthermia (HPT). PDT consists of therapeutic approaches involving the activation of photosensitized drugs by a visible light source, always associated with the generation of cytotoxic reactive oxygen species (ROS) and other free radicals, by type I or type II photochemistry pathway [8], to promote the selective destruction of target tissues [9–13]. An efficient photosensitizer should have a high absorption cross-section at a wavelength suitable for the desired application [14], normally close the maximum absorption wave-

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length of the drug in question, avoiding interference with natural endogenous molecules. PDT has been widely used to treat superficial tumors such as skin, head and neck tumors [15–17]. Zinc(II) phthalocyanine (ZnPc) is a promising second-generation photosensitizer for PDT that belongs to the phthalocyanine class due to its high optical absorption coefficient (in the range of 600–800 nm). These photosensitizers are insoluble in water, leading to self-aggregation in aqueous solutions, which drastically reduces the photosensitizing efficiency when used directly in biological fluids. To avoid these problems, an advanced drug delivery system is needed [9,18]. Several kinds of polymeric nanoparticles and polymerization methods have been used to encapsulate magnetic nanoparticles (MNPs) inside polymers [6,19–21]. MNPs present low toxicity and are promising materials for cancer treatment [22,23]. Additionally, induced hyperthermia as a procedure for cancer treatment involves raising the temperature of tumor tissues to 40–43 °C [24–26]. Solid tumors have been shown to have increased susceptibility to small temperature rises compared to healthy tissue due to their increased rate of cell cycling, increased hypoxia, reducing fluid exchange, increased acidity (pH 2–4) [25,26]. Therefore, the combination of hyperthermia with treatment methods like radiotherapy, chemotherapy and PDT turns these methods much more effective. The combination of HTP and PDT has produced promising results indicating synergistic interaction and significant tumor regression [17,27,28]. The heat released by HPT induces changes in cell cycle, causing faster denaturation of malignant cells through aggregation of nuclear proteins, thereby enhancing the sensitivity of cells denatured previously with PDT [29].

The simultaneous encapsulation of MNPs and ZnPc in the same system with controlled release is a promising new option for cancer treatment. Thus, the objectives of this work were to encapsulate MNPs and ZnPc in PMMA NPs by miniemulsion polymerization and to evaluate their *in vitro* effect in the presence of alternating magnetic field and/or light on human glioblastoma cells (U87MG). These cells cause the most aggressive brain tumors, with low life cure rates after surgery associated with traditional therapies. Hence, this investigation can lead to promising new cancer treatments.

2. Materials and methods

2.1. Materials

For the synthesis of magnetic nanoparticles (MNPs) coated with oleic acid (OA) the following reagents were used: ferrous sulfate ($FeSO_4{\cdot}4H_2O$), iron(III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ammonium hydroxide (99%), and oleic acid (OA), all purchased from Vetec. For the synthesis of MNPsZnPc loaded PMMA NPs, the following reagents were employed: the monomer methyl methacrylate (MMA), purchased from Arinos; azobisisobutylonitrile (AIBN), sodium dodecyl sulfate (SDS), NaH_2PO_4 (sodium phosphate monobasic) and Na_2HPO_4 (sodium phosphate dibasic), purchased from Vetec; lecithin (Alpha Aesar); miglyol 812 (Sazol) and zinc(II) phthalocyanine (ZnPc) and *n*-methylpyrrolidone (NMP), both purchased from Sigma Aldrich. Distilled water was used throughout the experiments.

2.2. Synthesis of magnetic nanoparticles coated with oleic acid (MNPs(OA))

MNPs(OA) were prepared by the co-precipitation method. $FeCl_3{\cdot}6H_2O$ and $FeSO_4{\cdot}7H_2O$ (molar ratio of 1:1) were dissolved in a beaker containing distilled water under mechanical stirring at 800 rpm. Then, ammonium hydroxide was rapidly added to the solution. After 1 h, 20 mL of OA was added and the stirring continued (800 rpm) for another 30 min. Finally the MNPs(OA) solution was centrifuged and washed with ethanol three times to remove excess OA.

2.3. Synthesis of PMMA nanoparticles

The preparation of PMMA nanoparticles via miniemulsion polymerization was the same as described by Feuser et al. [30]. The aqueous phase was composed of 20 mL distilled water and the organic phase contained: 2 g of MMA (monomer), 0.1 g of lecithin (surfactant), 0.1 g of miglyol (costabilizer) and 0.04 g of AIBN (initiator). The organic phase was added dropwise under high shear using sonication with amplitude of 70% (Fisher Scientific, Sonic Dismembrator, 500 W). Sonication was maintained for 5 min (10 s on and 1 s off) in a beaker immersed in an ice bath to avoid the temperature increase during sonication. The miniemulsion was transferred to glass tubes (10 mL) at 70 °C where the polymerization took place for 3 h under protection from light. Afterwards, the material was cooled, centrifuged and washed several times with phosphate buffered saline solution (PBS) at pH 7.4. Subsequently nanoparticles were transferred to glass vials, frozen in liquid nitrogen and lyophilized (Lyophilizer, FreeZone 4.5-L Benchtop Freeze Dry System; Labconco, Kansas City, MO). The lyophilized powder was stored at room temperature (25 °C) before analysis.

2.4. Simultaneous encapsulation of MNPs and ZnPc in PMMA NPs by miniemulsion polymerization (MNPsZnPC-PMMA)

MNPsZnPc in PMMA NPs were prepared by miniemulsion polymerization. For encapsulation of MNPs and ZnPc, initially 1 mL of NMP containing 6 mg of ZnPc was added dropwise in a beaker containing 20 mL of distilled water (aqueous phase) under sonication with an amplitude of 70% (Fisher Scientific, Sonic Dismembrator, 500 W). Next, the organic phase (containing 2 g of MMA, 0.2 g of MNPs, 0.1 g of lecithin, 0.1 g of miglyol and 0.04 g of AIBN) was added dropwise under high shear to the previous aqueous ZnPc dispersion. The sonication was continued for 5 min (10 s on and 1 s off) in a beaker immersed in an ice bath to avoid temperature increase during sonication. The miniemulsion was transferred to glass tubes (10 mL) at 70 °C, where the polymerization took place for 3 h under protection from light. Afterwards, the material was cooled, centrifuged and washed several times with PBS at pH 7.4. Subsequently, NPs were transferred to glass vials, frozen in liquid nitrogen and lyophilized (FreeZone 4.5-L Benchtop Freeze Dry System; Labconco, Kansas City, MO). The lyophilized powder was stored at room temperature (25 °C) before analysis.

2.5. Characterization

Monomer consumption was measured by gravimetric analysis of samples withdrawn from the polymerization medium at different times and the reaction was stopped with the addition of 1% (w/w) hydroquinone solution. The determination of the residual monomer concentration after the miniemulsion polymerization was performed by GC (Shimadzu GC2010AF). Particle size, polydispersity index (PdI) and surface charge were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS analyzer. All samples were analyzed five times, from which we calculated the average and standard deviation (SD). The NPs morphology was observed using a JEOL, JEM 2100F, transmission electron microscope (TEM) operated at 100 kV. For this analysis, several drops of the diluted sample were placed on a 200 mesh Formvar/carbon copper grid (Electron Microscopy Science). X-ray diffraction (XRD) experiments were performed to identify the crystallographic structure of free ZnPc, MNPs and MNPsZnPc-PMMA NPs. The crystalline phase of NPs was identified by XRD measurements using a XPert-

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