

Glutaric acid as a spacer facilitates improved intracellular uptake of LHRH–SPION into human breast cancer cells

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Abstract: Superparamagnetic iron oxide nanoparticles (SPIONs) bound directly to luteinizing hormone releasing hormone (LHRH) have shown high efficiency for intracellular uptake to breast cancer cells, MDA-MB-435S.luc. We demonstrate in this communication that inclusion of a small spacer molecule such as glutaric acid (Glu) in between SPION and LHRH increases further receptor mediated intracellular uptake. LHRH-bound SPIONs with and without the spacer molecule were nontoxic.

Keywords: Glutaric acid; luteinizing hormone releasing hormone; superparamagnetic iron oxide nanoparticles, MDA-MB-435S.luc., intracellular uptake

Introduction

There is a growing interest in developing superparamagnetic nanoparticle-based materials that are biocompatible, biodegradable, and efficient in intracellular uptake to cancer cells for applications in cancer therapy and diagnosis (Majumdar et al 1989; Weissleder et al 1990; Josephson et al 1998; Huh et al 2005; Song et al 2005; Alexiou et al 2006; Zhou et al 2006). While there is a continuous progress in this direction in terms of excellent experimental and theoretical work, there are several challenges that need to be addressed (Soppimath et al 2001; Sapra and Allen 2003; Gabizon et al 2004; Matsumura et al 2004; Yoo and Park 2004). One of the most important challenges is to prevent uptake of nanoparticles by the reticuloendothelial system (RES) and enhance their circulation times so that they can navigate through irregularities of tumoral vasculature leading to efficient intracellular uptake preferably via endocytosis. The efficacy of a nanoparticulate system is determined by their ability for intracellular uptake in *in vitro* studies. Experimental studies, mathematical models, and computer simulations have already demonstrated that rapid intracellular uptake can be improved through suitable surface modifications, especially coating of superparamagnetic iron oxide nanoparticles (SPIONs) with hydrophilic polymers and surfactants (Jeon et al 1991; Lasic et al 1991; Torchlin et al 1994; Alexiou et al 2006). Several studies have demonstrated that polyethylene glycol (PEG) coatings, due to their biocompatibility, resistance to proteins, and lack of antigenicity, are the most widely utilized for increasing circulation times and enhancing intracellular uptake of SPIONs in cancer cells (Zhang and Zhang 2005). Dextran-coated SPIONs also have been demonstrated to improve intracellular uptake (Hogemann et al 2000; Berry et al 2004; Funovics et al 2004). In addition to simple coatings such as PEG and dextran, there are also investigations related to more complex coatings involving multi-step synthetic procedures to prevent RES uptake and improved cellular uptake (Kohler et al 2005, 2006; Neuberger et al 2005; Woo and Hong 2005; Koeseoglu 2006). However, to improve production and *in vivo* applications, it is important to avoid complicated and lengthy chemical procedures for SPION coatings. We recently demonstrated that ligands such as luteinizing hormone releasing hormone (LHRH) act as targeting

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agents for breast cancer cells that express receptors for LHRH (Leuschner et al 2003). We have also demonstrated that intracellular accumulation of LHRH-bound SPIONs (without any PEG or dextran coating on SPIONs) in MDA-MB-435S. luc cells *in vitro* was 12-fold higher than the corresponding free SPIONs uptake (Leuschner et al 2005, 2006; Zhou et al 2006). The results are not very surprising as LHRH is a decapeptide and can function as coating (in addition to being a targeting agent) thus preventing macrophage recognition, RES uptake, enhancing circulation time, and facilitating cellular uptake.

In addition to demonstrating the concept of utilizing a targeting agent to act as a coating for improved intracellular uptake, we are also currently investigating the introduction of a small spacer between SPION and LHRH to engineer SPION conjugates for further improvements in intracellular uptake. In this study, we have synthesized SPIONs and covalently bound glutaric acid (Glu) to SPIONs followed by LHRH using carbodiimide chemistry. We present below details of synthesis, characterization, intracellular uptake, and cytotoxicity of newly engineered SPIONs and compare the data with that of free SPIONs and SPION-LHRH.

Experimental

Iron II chloride ($\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$) 98%, iron III chloride (FeCl_3) 97%, ammonium hydroxide (NH_4OH) 29.05%, 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide hydrochloride (EDC), and Glu were purchased from Sigma Aldrich (St. Louis, MO, USA). Air-free nanopure water was made in the lab by refluxing nanopure water, made with a Barnstead Nano-Pure Water System (Barnstead International, Dubuque, IA, USA), under inert atmosphere. During the synthesis of the nanoparticles a 750D Sonicator (VWR International, Leuven,

Belgium) was used, as well as a 1160A PolyScience Chiller (VWR International).

The SPIONs used in this study were prepared using the procedure similar to the one reported by Kumar and colleagues (2004). For the covalent attachment of Glu to the SPIONs, 60 mg of magnetite nanoparticles were dispersed in 6 ml of water using a sonication bath at room temperature for fifteen minutes. A solution of 42 mg carbodiimide and 1.5 ml water was added. The mixture was sonicated for 10 more minutes and then cooled to 4 °C in a chiller. A solution of 3.7 mg Glu in 1.5 ml of water was added, and the reaction temperature was maintained at 4 °C for 2 h. The particles were then allowed to settle on a permanent magnet. The supernatant was removed and the particles were washed three times with water, twice with ethanol, and dried under nitrogen.

The above procedure was followed for the functionalization of the glutaric acid-bound SPIONs with LHRH. Substituting only 3.7 mg of LHRH instead of Glu and 60 mg of Glu-SPIONs instead of plain magnetite.

The supernatant of the SPION-Glu particles and of the SPION-Glu-LHRH particles was analyzed by high-performance liquid chromatography (HPLC) for the presence of unbound LHRH or Glu, according to which binding had taken place. The size and morphology of the magnetic nanoparticles were observed using a JOEL 100X (JEOL Ltd., 1-2, Musashino 3-chome Akishima Tokyo, Japan) transmission electron microscopy (TEM) at 80kV. The mean diameter was estimated from 300 particles with the aid of MetaVue software (Molecular Devices Corporation, Downingtown, PA, USA). Binding of Glu and LHRH was confirmed by Fourier transform infrared (FTIR) spectra obtained using a Thermo Nicolet Nexus 6/870 FTIR (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Samples for FTIR analysis were

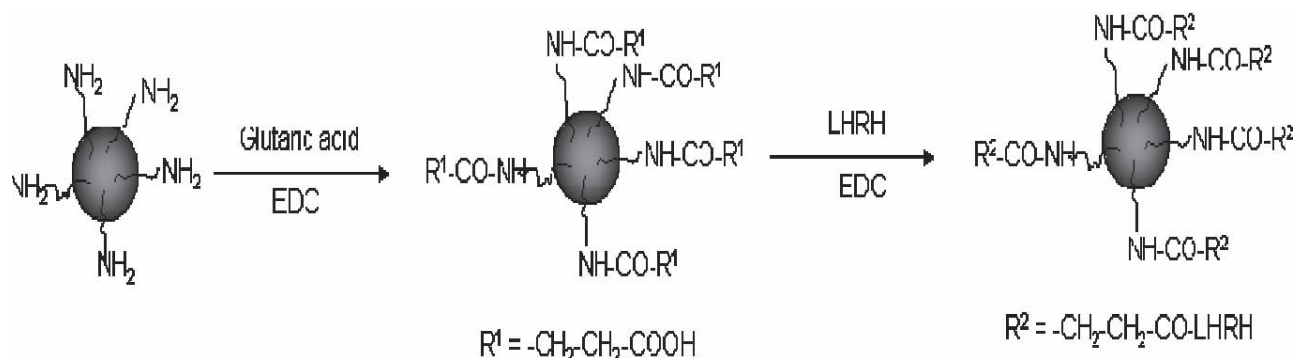


Figure 1 Schematic representation of step-wise binding of glutaric acid and LHRH to SPIONs.

Abbreviations: EDC, N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride; LHRH, luteinizing hormone releasing hormone; SPIONs, superparamagnetic iron oxide nanoparticles.

prepared using KBr pellets. The magnetic properties of the SPION–Glu–LHRH particles were studied with a Quantum Design MPMS-5S SQUID magnetometer (Quantum Design, San Diego, CA, USA).

MDA-MB-435S human breast cancer cells were obtained from the American Type Culture Collection (Rockville, MD) and cultured as described previously (Leuschner et al 2003). MDA-MB-435S cells were transfected with exogenous DNA by lipofection using the plasmid pRC/CMV-luc containing the *Photinus pyralis* luciferase gene and an antibiotic resistance gene under transcription control of the cytomegalovirus promoter (Rubio et al 1998, 2000). MDA-MB-435S.luc cells (60,000 cells/well) were grown in 24 well plates and incubated with LHRH–SPION, LHRH–Glu–SPION or SPION (Fe 1 mg/ml) at 37 °C for 3 h in the presence and absence of LHRH (50 µM). At the end of the incubations the cells were detached from the culture plates, washed, centrifuged and the cell pellets were resuspended in HCl (1 M). Iron contents were determined spectrophotometrically using a Prussian Blue reaction kit.

Toxicity studies were conducted in cultures of MDA-MB-435S.luc cells using mitochondrial dependent formazan formation (MTT) assay as described by Berridge and Tan (1993).

Results and discussion

The binding of LHRH and Glu to the SPION particles is facilitated by the presence of amine groups on the surface of

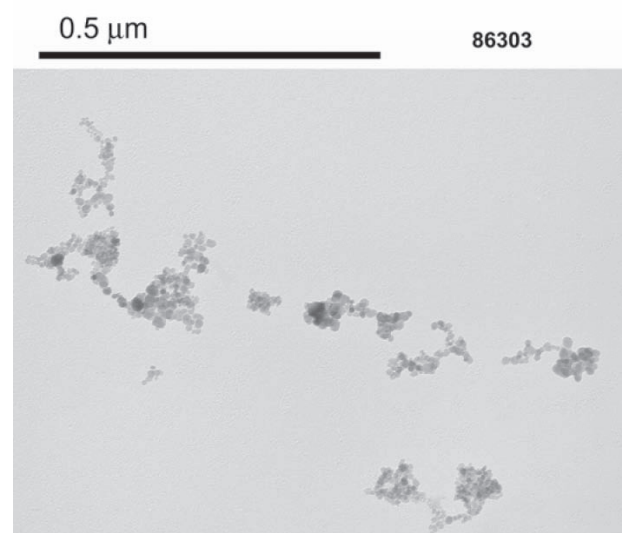


Figure 2 A TEM micrograph of SPION–Glu–LHRH nanoparticles.

Abbreviations: Glu, glutaric acid; LHRH, luteinizing hormone releasing hormone; SPIONs, superparamagnetic iron oxide nanoparticles; TEM, transmission electron microscopy.

the SPIONs (Kumar et al 2004; Shieh et al 2005). The synthetic strategy adopted is shown in Figure 1. Analysis of the supernatant after the binding process by HPLC is one way to determine that binding has taken place. HPLC analysis did not reveal the presence of any free Glu or LHRH, confirming that the biomolecules have been bound to the SPIONs. The size and morphology of SPION–Glu–LHRH nanoparticles

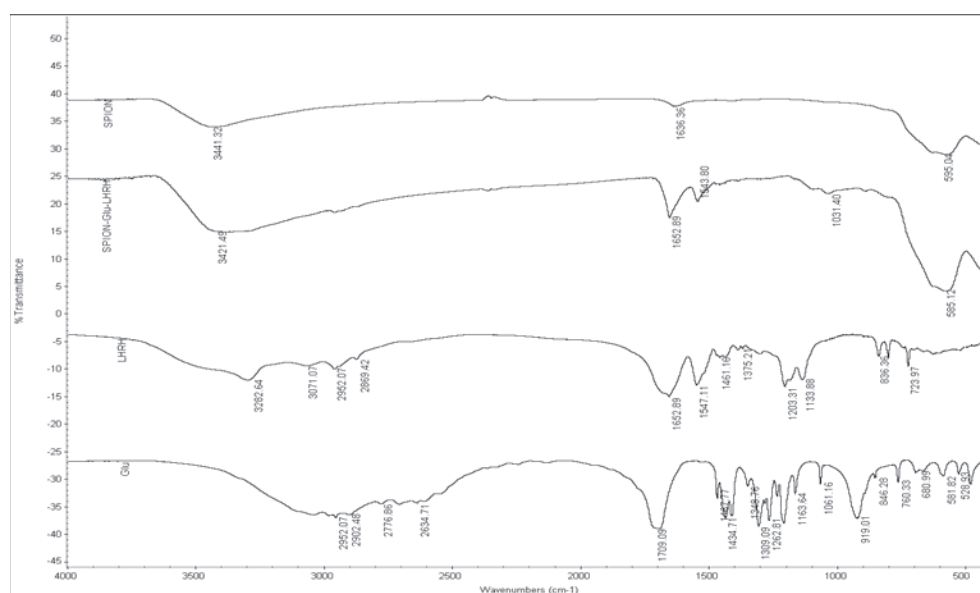


Figure 3 FTIR spectra of SPION, SPION–Glu, and SPION–Glu–LHRH.

Abbreviations: FTIR, Fourier transform infrared; Glu, glutaric acid; LHRH, luteinizing hormone releasing hormone; SPIONs, superparamagnetic iron oxide nanoparticles.

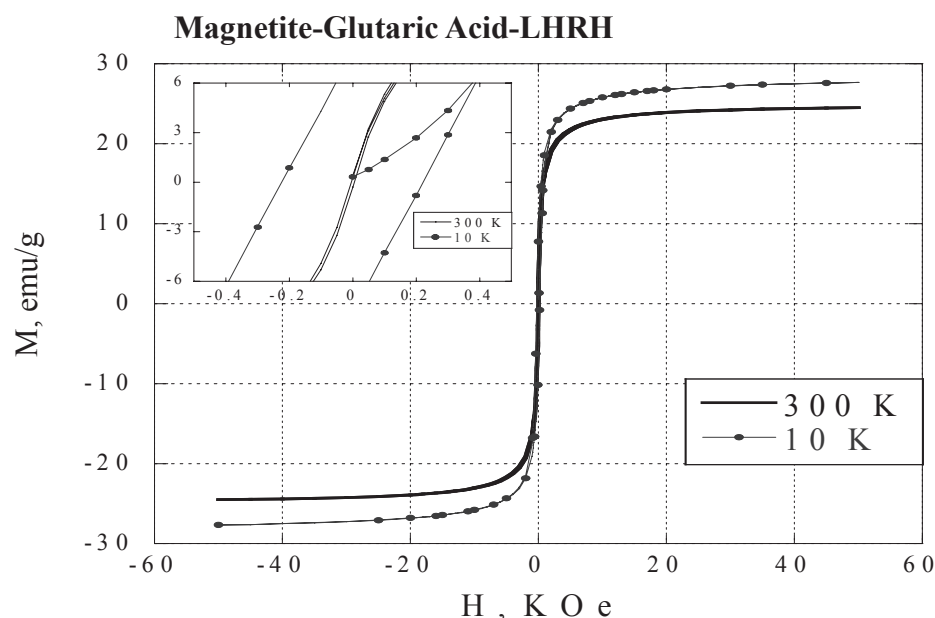


Figure 4 Magnetic properties of LHRH-Glu-SPIONs.

Abbreviations: Glu, glutaric acid; LHRH, luteinizing hormone releasing hormone; SPIONs, superparamagnetic iron oxide nanoparticles.

was investigated using TEM. A TEM micrograph of the nanoparticles is shown in Figure 2. The particles are roughly spherical in shape with a mean diameter of 9.12 nm. FTIR was also utilized to characterize SPION–Glu–LHRH nanoparticles. The particles exhibited typical amide bond signatures at around 1652 cm^{-1} and the absence of carboxylic carbonyl stretching of glutaric acid at 1709 cm^{-1} (Figure 3).

The SPION–Glu–LHRH nanoparticles are superparamagnetic as demonstrated by weak hysteresis in the plots of magnetization versus magnetic field (M-H loop) at 10K (Figure 4). Compared with free SPIONs, SPION–Glu–LHRH nanoparticles showed lower saturation magnetization ($M_s = 25\text{ Vs } 72.1\text{ emu/g}$). The reduction in M_s on binding Glu–LHRH to the SPIONs is not very surprising due to quenching of magnetic moment through electron exchange between organic molecules and surface atoms (Vanleeuwen et al 1994).

In vitro analysis of the potency for cellular uptake was carried out using breast cancer cells, MDA-MB-435S.luc that expresses LHRH-receptors. The results are shown in Figure 5. The results demonstrate that LHRH–SPION and LHRH–Glu–SPION specifically and more effectively accumulate in the breast cancer cells, reaching $82.3 \pm 25\text{ pg/cell}$ for SPIONs, $133 \pm 17\text{ pg/cell}$ for Glu–SPIONs ($p < 0.003$ vs SPION), $165 \pm 16\text{ pg/cell}$ for LHRH–SPIONs ($p < 0.003$ vs SPION) and $223 \pm 16\text{ pg/cell}$ with LHRH–Glu–SPION ($p < 0.05$ vs Glu–SPION, $p < 0.002$ vs LHRH–SPION). This accumulation is significantly reduced in the presence of the same

ligand LHRH for LHRH–SPION and LHRH–Glu–SPION ($106 \pm 26\text{ pg/cell}$, $123 \pm 25\text{ pg/cell}$, respectively, $p < 0.001$). This observation suggests that iron uptake was driven through receptor mediated endocytosis. The introduction of a spacer significantly increased the intracellular iron uptake from 165

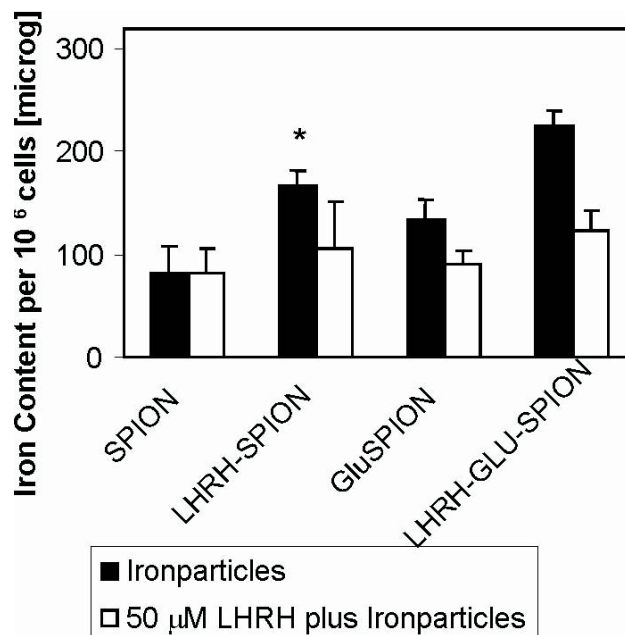


Figure 5 Intracellular uptake of LHRH–Glu–SPIONs.

Abbreviations: Glu, glutaric acid; LHRH, luteinizing hormone releasing hormone; SPIONs, superparamagnetic iron oxide nanoparticles.

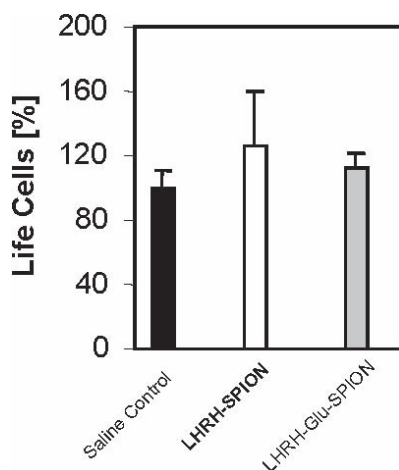


Figure 6 Toxicity of LHRH-Glu-SPIONs.

Abbreviations: Glu, glutaric acid; LHRH, luteinizing hormone releasing hormone; SPIONs, superparamagnetic iron oxide nanoparticles.

to 223.3 pg/cell, $p < 0.001$. Toxicity studies over a period of 5 days revealed that none of the three iron oxide conjugates were toxic to human breast cancer cells (Figure 6).

In summary, the work reported indicates that incorporation of a small spacer molecule such as Glu between LHRH and SPION shows significant enhancement in intracellular uptake without causing toxicity to the breast cancer cells.

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