Pluronic/chitosan shell cross-linked nanocapsules encapsulating magnetic nanoparticles

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Abstract—We have developed novel Pluronic/chitosan nanocapsules encapsulating iron oxide nanoparticles. These nanocapsules were produced by dispersing hydrophobically-modified iron oxide nanoparticles and amine-reactive Pluronic derivatives in an organic solvent, and subsequently emulsification in an aqueous chitosan solution by ultrasonication. The resultant shell cross-linked nanocapsules had a unique core/shell type nanoreservoir architecture: an inner core encapsulating magnetic nanoparticles and a hydrophilic Pluronic/chitosan polymer shell layer, as confirmed by thermogravimetric analysis and transmission electron microscopy. Confocal laser scanning microscopy revealed that the rhodamine-labeled nanocapsules were efficiently internalized by human lung carcinoma cells upon exposure to an external magnetic field. The present study suggested that these novel nanomaterials could be dually utilized for the magnetically-triggered delivery of various anti-cancer agents and for cancer diagnosis with magnetic resonance imaging.

Key words: Pluronic; chitosan; nanocapsules; magnetism; drug-delivery systems.

INTRODUCTION

Over the past decades, various inorganic nanoparticles, such as gold and iron oxide nanoparticles, and quantum dots have attracted considerable attention in the field of nanobiotechnology due to their unique and controllable optical, magnetic and electronic properties [1–3]. Among them, superparamagnetic iron oxide nanoparticles have been extensively studied for diverse biomedical applications, including ultra-sensitive detection of DNA and proteins [4, 5], magnetic drug targeting [6], hyperthermal therapy [7] and magnetic resonance imaging (MRI) [8, 9]. Particularly in cancer therapeutics, magnetic nanoparticles open new opportunities for the

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development of efficient drug carriers that can deliver anti-cancer agents specifically into cancerous tissues by a magnetic field [10]. However, it is crucial that the surface of the magnetic nanoparticles should be suitably tailored for enhanced colloidal stability, biocompatibility with cells or tissues, and conjugation of cancer-targeting ligands. Several approaches have been employed to stabilize and functionalize the magnetic nanoparticles, including covalent attachment of hydrophilic polymers [11, 12], surface-initiated growth of polymer shells [13], microemulsion polymerization [14] and self-assembly of amphiphilic co-polymers into micelles [15] around the nanoparticles.

Pluronic tri-block co-polymers are non-ionic macromolecular surfactants composed of poly(oxyethylene)-block-poly(oxypropylene)-block-poly(oxyethylene) (PEO-PPO-PEO). It is known that they self-associate to form spherical micelles in aqueous solution above a certain micelle temperature by hydrophobic interactions between the PPO middle blocks [16–18]. We have previously developed a series of Pluronic nanocapsules by shell cross-linking of Pluronic co-polymers with heparin, polyethylenimine, or gold nanoparticles at an organic/aqueous interface [19–21]. These nanocapsules exhibited a dramatic volume transition over a broad temperature range by thermally-triggered micellization of cross-linked or grafted Pluronic co-polymer chains in the interior. Furthermore, we demonstrated recently that a covalently cross-linked polymer shell of the nanocapsules could efficiently encapsulate hydrophobic oil droplets, evolving into a nanoreservoir for water insoluble anti-cancer drugs [22].

In the present work, Pluronic/chitosan nanocapsules encapsulating iron oxide nanoparticles in their nanoreservoir structure were produced for magnetically triggered intracellular delivery of various therapeutic agents. Chitosan is a natural polysaccharide composed of glucosamine and N-acetyl-D-glucosamine, and has been widely exploited as a promising gene carrier due to its highly cationic, non-cytotoxic and biodegradable nature [23–25]. To synthesize Pluronic/chitosan shell cross-linked nanocapsules functionalized with magnetic nanoparticles, a mixture of hydrophobically-modified iron oxide nanoparticles and amine-reactive Pluronic derivatives in an organic solvent was ultrasonically emulsified in an aqueous chitosan solution. The cross-linking reactions at emulsion interfaces led to the immediate formation of Pluronic/chitosan composite shell layer, while encapsulating iron oxide nanoparticles within the core. Importantly, these novel nanocapsules are expected to enhance the intracellular delivery of loaded therapeutic agents, such as plasmid DNA and oligonucleotides, in response to an external magnetic field. We investigated the structure and morphology of the nanocapsules by thermogravimetric analysis (TGA) and transmission electron microscopy (TEM), and their magneto-responsible cellular uptake behavior was observed by confocal laser scanning microscopy (CLSM).
MATERIALS AND METHODS

Materials

Pluronic F127 ((PEO)_{100}(PPO)_{65}(PEO)_{100}, M_w = 12.6 \times 10^3) was obtained from BASF (Parsippany, NJ, USA). Water-soluble chitosan oligosaccharide (M_w = 2000, deacetylation degree 96%) was supplied by KITTOLIFE (Seoul, South Korea). Ferric chloride hexahydrate (FeCl_3 \cdot 6H_2O), ferrous chloride tetrahydrate (FeCl_2 \cdot 4H_2O), ammonium hydroxide, oleic acid and p-nitrophenyl chloroformate (p-NPC) were purchased from Sigma (St. Louis, MO, USA). 5,6-Carboxytetramethylrhodamine succinimidyl ester (NHS-rhodamine) was purchased from Pierce Biotechnology (Rockford, IL, USA). All other chemical reagents were of analytical grade.

Synthesis of oleic-acid-stabilized iron oxide nanoparticles

Oleic-acid-stabilized iron oxide nanoparticles were synthesized by using a coprecipitation method reported previously [26]. In brief, ferric chloride hexahydrate (810.9 mg, 3 mmol) and ferrous chloride tetrahydrate (298.2 mg, 1.5 mmol), dissolved in 45 ml deionized water, were placed in a 150-ml round-bottomed flask equipped with a stirrer. Ammonium hydroxide (5 M, 3 ml) was added in a dropwise manner over 1 min into the above solution, to which 0.28 ml (0.99 mmol) of oleic acid was slowly added under vigorous stirring. The reaction was carried out at room temperature for 20 min under nitrogen atmosphere. After heating up to 80°C for 30 min to evaporate ammonia, the black precipitate was isolated from the solvent by magnetic decantation, washed three times with 30 ml of nitrogen-purged water and then lyophilized.

Synthesis of Pluronic/chitosan shell cross-linked nanocapsules encapsulating magnetic nanoparticles

Pluronic/chitosan nanocapsules encapsulating magnetic nanoparticles were prepared by using a modified emulsification/solvent evaporation method [19–22]. Pluronic F127 co-polymer was first pre-activated with p-NPC at its two terminal hydroxyl groups as previously described [21]. Oleic-acid-stabilized iron oxide nanoparticles were dispersed in dichloromethane at a concentration of 10 mg/ml by sonication for 5 min. 200 µl of the dispersion was mixed with 6 mg of the activated Pluronic co-polymer. The above mixture was added dropwise to an aqueous solution (10 ml, pH 10) containing 4 mg of chitosan oligosaccharides. The solution was sonicated for 3 min using a Branson sonifier 450 (20 kHz, output control 3, duty cycle 40%) and then neutralized by HCl to terminate the reaction. The oil-in-water emulsion solution was quickly transferred to a rotary evaporator with a water bath set at 40°C to remove residual dichloromethane. The resultant solution was dialyzed against deionized water by a Spectra/Por dialysis membrane with a MW cutoff of 50 \times 10^3 and subsequently concentrated by magnetic decantation.
Characterization of Pluronic/chitosan shell cross-linked nanocapsules encapsulating magnetic nanoparticles

The hydrodynamic diameters of iron oxide nanoparticle-embedded Pluronic/chitosan nanocapsules were measured by using a dynamic light scattering instrument (Zeta-Plus, Brookhaven, NY, USA) equipped with a HeNe laser at a wavelength of 632 nm. The measurement was carried out in triplicate at a concentration of 0.3 mg/ml at 37°C. For thermogravimetric analysis, lyophilized samples were placed in aluminum sample cells, and a thermogram for each sample was obtained using a TG 209 F3 Tarsus thermo-microbalance (NETZSCH Instruments, Burlington, MA, USA). Samples were heated at a rate of 10°C/min up to 500°C under a flow of nitrogen gas. X-ray powder diffraction profiles were measured using Rigaku D/max-IIIC diffractometer with CuKα radiation (40 kV, 80 mA). Surface morphology and internal structure of the nanocapsules were visualized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Thirty microliter of the nanocapsule solution (0.3 mg/ml) was equilibrated and dried on a freshly prepared carbon tape at 37°C, and then its image was obtained with a Philips 535M scanning electron microscope. The same amount of the solution was deposited onto a 300 mesh Formvar/carbon-coated copper grid and then observed under a Philips F20 transmission electron microscope. The average particle size was determined by measuring the diameter of more than 20 particles in the SEM and TEM images.

Evaluation of magnetically triggered intracellular uptake of nanocapsules

Green fluorescent protein-expressing human lung carcinoma A549 (GFP-A549) cell line was obtained from Samyang (Seoul, South Korea). Pluronic/chitosan nanocapsules encapsulating magnetic nanoparticles were fluorescently labeled with rhodamine, in order to visualize their intracellular uptake into GFP-A549 cells. Briefly, the nanocapsules were dispersed in phosphate-buffered saline (pH 7.4) solution, to which 2 mg (3.8 µmol) of NHS-rhodamine dissolved in dimethyl sulfoxide was slowly added. After 12 h of reaction at 4°C in a dark room, the resultant solution was dialyzed against deionized water by a Spectra/Por dialysis membrane with a MW cutoff of 50 × 10³ and purified by magnetic decantation to remove excess rhodamine dyes. GFP-A549 cells were plated over a cover slide on a 60 mm Petri dish at a density of 5 × 10⁵ cells per dish and cultivated for 24 h at 37°C. After treatment of rhodamine-labeled nanocapsules (0.1 mg/ml), the cells were incubated for 30 min, 1 h and 2 h at 37°C either with or without exposing to an exterior neodymium magnet. After a specified incubation period, cells were washed with PBS, and then fixed with 4% (w/v) formaldehyde solution in PBS. The cells were examined by using a LSM510 confocal laser scanning microscope (Carl Zeiss, Germany) equipped with a 488 nm Argon laser and 543 nm HeNe laser.
RESULTS AND DISCUSSION

Oleic-acid-stabilized iron oxide nanoparticles were initially prepared by chemical co-precipitation of ferric and ferrous ions in the presence of oleic acid on addition of ammonium hydroxide. Since carboxylic acid of oleic acid was chemisorbed onto the surface of \textit{in situ} formed iron oxide crystals, iron oxide nanoparticles were hydrophobically modified with long oleic chains to become dispersible in organic solvents. Oleic acid with a concentration of 41 wt\% with respect to the total formulation weight was chosen to prepare the iron oxide nanoparticles, because it was reported previously that the amount of oleic acid at that concentration was required for optimally dispersible iron oxide nanoparticles in hexane [26]. The resultant oleic acid-stabilized iron oxide nanoparticles could be obtained as black dry powder and then easily dispersed in various non-polar organic solvents such as hexane, chloroform and dichloromethane with ultrasonic treatment. The mean diameter of these nanoparticles estimated by TEM was $10.4 \pm 3.1$ nm, which was consistent with the previous results [14, 26].

Figure 1 schematically illustrates the synthesis of Pluronic/chitosan shell cross-linked nanocapsules encapsulating magnetic nanoparticles (PM nanocapsules). Oleic acid-stabilized iron oxide nanoparticles and $p$-NPC-activated Pluronic copolymers were mixed together in dichloromethane and dropped into aqueous chitosan solution. The solution was immediately subjected to ultrasonication for 3 min to form a stable oil-in-water emulsion where the oleic-acid-stabilized iron oxide nanoparticles were entrapped inside oil droplets but not located in a

![Figure 1. Synthetic scheme of magnetic nanoparticle functionalized Pluronic/chitosan shell cross-linked nanocapsules (PM nanocapsules). This figure is published in colour at http://www.ingenta.com](http://www.ingenta.com)
continuous aqueous phase [27, 28]. As the terminal \( p \)-NPC groups of the Pluronic co-polymers reacted with primary amine groups in chitosan oligosaccharides to form covalent amide linkages, Pluronic co-polymers and chitosan were chemically cross-linked at the organic/aqueous interface of the emulsion droplets. Once the residual solvent within the droplets was evaporated under reduced pressure, Pluronic/chitosan shell cross-linked nanocapsules encapsulating the iron oxide nanocapsules were successfully produced. Since these nanocapsules were expected to exhibit magneto-responsive mobility along with intrinsic functions of chitosan oligosaccharides, it was hypothesized that they would interact electrostatically with negatively charged therapeutic agents such as plasmid DNA and oligonucleotides, and then enhance the intracellular delivery of the loaded drugs in response to an external magnetic field.

We performed dynamic light scattering (DLS) to elucidate the size distribution and dispersion stability of PM nanocapsules in aqueous solution. As shown in Fig. 2, monodisperse PM nanocapsules were produced with a hydrodynamic diameter of \( 258.3 \pm 18.3 \) nm at \( 37^\circ C \). It should be noted that the dispersion stability of oleic acid-stabilized iron oxide nanoparticles were greatly changed as they were encapsulated into Pluronic/chitosan nanocapsules (inset photograph). While the oleic-acid-stabilized iron oxide nanoparticles were well dispersed in dichloromethane, they were unable to disperse in deionized water and finally precipitated out of solution presumably because they interacted with each other via hydrophobic interactions.

**Figure 2.** Hydrodynamic diameter of PM nanocapsules. The inset photograph shows the dispersion of oleic-acid-stabilized iron oxide nanoparticles in (a) dichloromethane, (b) deionized water and (c) dispersion of PM nanocapsules in deionized water. This figure is published in colour at http://www.ingenta.com
to form larger insoluble aggregates. However, it can be seen that the aqueous dispersion of PM nanocapsules yield a transparent brown solution without showing any precipitates, indicative of superior stability of the nanocapsules. This result suggested that the oleic-acid-stabilized iron oxide nanoparticles were completely encapsulated within a cross-linked Pluronic/chitosan composite shell, leading to the formation of hydrophilic and dispensible nanocapsules in aqueous solution.

The composition of oleic-acid-stabilized iron oxide nanoparticles and PM nanocapsules was investigated by thermogravimetric analysis (TGA). The thermogram in Fig. 3 revealed that the thermal decomposition of oleic acid occurred over a broad range of temperature from 200°C to 500°C. While the weight loss for Pluronic co-polymer took place mainly at 380°C, chitosan oligosaccharide was gradually decomposed as the temperature increased from 100°C to 500°C (not shown here). Since almost all the polymers in PM nanocapsules were completely decomposed at 500°C, the magnetite content could be calculated based on the remaining weight. The weight loss curve demonstrated that the magnetite content of the oleic acid-stabilized iron oxide nanoparticles and PM nanocapsules was about 77.0 and 66.4 wt%, respectively. The high magnetite content of PM nanocapsules would be advantageous for magnetic drug targeting applications because it renders them to rapidly respond to a magnetic field.

X-ray diffraction pattern (XRD) analysis was employed to confirm the formation of crystalline magnetite nanoparticles. As shown in Fig. 4, both oleic-acid-stabilized iron oxide nanoparticles and PM nanocapsules showed characteristic diffraction peaks corresponding to the (220), (311), (400), (511) and (440) planes of magnetite.
Figure 4. X-ray diffraction pattern of (A) oleic-acid-stabilized iron oxide nanoparticles and (B) PM nanocapsules.

A face centered cubic (fcc) lattice of magnetite crystals [12, 26]. Since the shell cross-linking reaction occurred primarily at the interface of the oil/water emulsion droplets containing the iron oxide nanoparticles, it was conceivable that the encapsulation process would not affect the crystalline structure and magnetization property of the iron oxide nanoparticles. Therefore, it is safe to say that the current encapsulation process is potentially applicable for stabilization and functionalization of a variety of inorganic nanomaterials which are poorly soluble and processable in aqueous solution. The average size of the iron oxide nanoparticles calculated from the half width of (311) reflection by the Scherrer formula [29] was 6.9 nm, similar to those obtained from TEM imaging.
Figure 5. (A, B) SEM and (C, D) TEM images of PM nanocapsules. Samples were equilibrated and deposited onto a freshly prepared carbon tape or a 300 mesh carbon-coated copper grid at 37°C. Each pair of images was taken at a different magnification.

The size and surface morphology of PM nanocapsules were characterized by scanning electron microscopy (SEM). As shown in the SEM images (Fig. 5A and 5B), PM nanocapsules were round shaped with an average diameter of 221.3 ± 120.2 nm. It was found that these nanocapsules had a well-dispersed structure without significant aggregation, indicating that they were effectively stabilized by a cross-linked hydrophilic polymer shell in aqueous solution. It was likely that the dispersion stability was partially attributed to the charge repulsion phenomena exerted by cationic chitosan oligosaccharides cross-linked or grafted on the nanocapsules [30]. The internal structure of PM nanocapsules was directly visualized by transmission electron microscopy (TEM). In the TEM images (Fig. 5C and 5D), it was clearly observed that iron oxide nanoparticles with an average diameter of around 10 nm (shown as dark spots) were formed into a larger spherical aggregate. From this distinct architecture, it was confirmed that the iron oxide nanoparticles were successfully encapsulated in the interior cavities of the nanocapsules. This implies that the covalently cross-linked polymer framework surrounding the iron oxide nanoparticles protected them from leaking out of the nanocapsules efficiently. Physical entrap-
ment and/or hydrophobic interactions between the long oleic chains and PPO segments of Pluronic co-polymers might play an important role in the successful encapsulation of the iron oxide nanoparticles [22]. Furthermore, the average diameter (205.2 ± 122.3 nm) of the nanocapsules estimated by TEM was in agreement with those determined from DLS and SEM results. Hence, TEM analysis demonstrated a unique nanoreservoir structure of PM nanocapsules capable of enclosing diverse inorganic nanomaterials in the interior.

We also investigated the effect of an external magnetic field on the mobility of PM nanocapsules. As shown in Fig. 6, PM nanocapsules were rapidly captured by a magnet and completely separated from the solution after only 20 min. This revealed that the iron oxide nanoparticles retained their magnetic property after they were encapsulated within the nanocapsules. The magneto-responsible properties of these nanocapsules suggest the potential applicability for magnetic drug targeting to increase the concentration of therapeutic agents in a target site exposed to a magnetic field.

To prove the capability of PM nanocapsules as efficient drug carriers, we examined their intracellular uptake into green fluorescent protein-expressing human lung carcinoma A549 (GFP-A549) cells by confocal laser scanning microscopy. PM nanocapsules were fluorescently labeled with rhodamine, in order to visualize in sharp contrast their subcellular distribution. Figure 7 illustrates the confocal microscopic images of GFP-A549 cells following incubation with rhodamine-labeled PM nanocapsules at a concentration of 0.1 mg/ml. It was found that these nanocapsules appearing as red fluorescent dots were not efficiently internalized by the cells, even after incubation for 2 h without a magnetic field. In contrast, strong red fluorescence was already detected within the cell cytoplasm only after 30 min exposure to a exterior magnet, indicating that PM nanocapsules were rapidly taken up by the cells under a magnetic field. It is noteworthy that the cellular uptake

Figure 6. Photographs showing magnetic capture of PM nanocapsules. This figure is published in colour at http://www.ingenta.com
of these nanocapsules gradually increased with continual exposure to a magnet. Since the nanocapsules did not present any cellular targeting moiety on surface, their cellular uptake was probably mediated by non-specific absorptive endocytosis [31, 32]. However, the magnetic field would accelerate the sedimentation of PM nanocapsules on the cell surface, thereby increasing contact with the target cells [10, 33]. Thus, application of the magnetic field could lead to rapid and efficient
delivery of various therapeutic agents complexed with magnetic nanoparticles to the desired cells or tissue. Consequently, we demonstrated that these novel polymer nanocapsules encapsulating magnetic nanoparticles would be potentially applied for magnetically triggered delivery of various therapeutic agents and early detection of life-threatening diseases with magnetic resonance imaging.

CONCLUSIONS

In summary, we present the development of magnetic nanoparticle functionalized Pluronic/chitosan nanocapsules for magnetically-triggered drug delivery. These nanocapsules were produced by shell cross-linking between Pluronic co-polymers and chitosan oligosaccharides at the interface of oil/water emulsion droplets containing hydrophobically modified iron oxide nanoparticles. The iron oxide nanoparticles were stably encapsulated within the hydrophilic shell layer of the nanocapsules. This encapsulation process did not disturb the magnetization properties of the encapsulated nanoparticles, suggesting its applicability as a novel strategy toward stabilization and functionalization of diverse inorganic nanomaterials. Moreover, the magnetic field significantly facilitated the cellular uptake of the rhodamine-labeled nanocapsules. These novel polymer nanocapsules functionalized with magnetic nanoparticles are expected to be widely used as efficient delivery vehicles for various therapeutic agents in response to an external magnetic field.

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REFERENCES