Syntehsis and evaluation of Gd3+ -Trp-PLGA as novel nanosized MR tumor imaging candidate

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ABSTRACT

Objective(s): Early detection of cancer can significantly increase the likelihood of successful treatment, and imaging assay can have a significant impact on cancer diagnosis. Although gadolinium compounds are used as a contrast agent in MRI, this substance has side effects and disadvantages. Nanotechnology has so far had a significant impact on medical imaging methods, especially MRI, nanoparticles are contrast enhancers that each with its characteristics have increased the quality of images and reduced toxicity.

Materials and Methods: In this study, a novel nano-conjugate based PLGA-tryptophan was synthesized and loaded with Gd3+ for using it as a potential MR imaging contrast agent to overcome the previous disadvantage. In vitro cell toxicity, cellular uptake and MR imaging parameters of the prepared nanoconjugate were investigated.

Results: The results showed no in vivo toxicity plus flowcytometry assays, good cellular uptake and large longitudinal (r1).

Conclusion: Convenient features of the nano-probe indicate that it is a promising agent to use as a MR imaging agent.

Keywords: Cell toxicity, Gd3+, MR imaging, PLGA, Trp, Relaxivity times

INTRODUCTION

Cancer is one of the primary causes of deaths world. Each year, millions of people are diagnosed with cancer all around the world and more than half of them who are diagnosed with cancer die from the disease [1]. Since, researchers have focused on synthesizing the novel carriers for targeting the cancer cells [2]. In recent years, synthesis of nanoparticles has gained more attention in the field of biomedicine. Nanoparticle synthesis is increased rapidly for their valuables properties such as, their large surface area compared to their volume ratio and the versatility of their surface chemistries [3]. Among various types of nanostructures, polymeric nanoparticles are more interesting for researchers because of their natural formation from natural resources and easy preparation process[4]. In addition, poly (lactic-co-glycolic acid) (PLGA) has attracted remarkable attention owing to its excellent biocompatibility, biodegradability and mechanical strength [4]. Because of these valuable properties, the current researches focus on the application of PLGA as a vehicle for the delivery of metals and MRI imaging agents. In this framework, Doiron et al, have reported PLGA as a carrier for imaging contrast agents. Results showed an elevation in MR and fluorescence contrasts[5]. Mariano and co-workers synthesized the PLGA based
nanoparticles for MR imaging. High stability and sensitivity of PLGA-NPs caused an increase in vivo uptake in murine melanoma xenograft as shown in MR imaging [6]. Furthermore, Liu et al. described Fe3O4-based PLGA nanoparticles as a MR contrast agent for in vivo detection of thrombosis. Toxicity studies revealed that these NPs are sufficiently nontoxic with good affinity for thrombosis [7]. Moreover, Schleich and his co-workers reported the dual anticancer iron oxide-loaded on PLGA-based nanoparticles for cancer therapy and MR imaging [8]. Amino acids represent an important class of nutrients for cancerous cells because of their high proliferation rate [9]. Recently, researchers have reported that amino acids have various benefits for selective delivery of therapeutic or diagnostic agents to the tumor site [10-12]. Tryptophan is an essential α-amino acid that plays many substantial roles in mammalian metabolism. Also, to the role of tryptophan in protein synthesis, it is linked to the regulation of immune tolerance and anti-tumor immune responses [13]. In addition, tryptophan works as a biochemical precursor for the serotonin [14], niacin [15], auxins [16].

The most important goal of this study was preparing the novel nanosized PLGA-tryptophan-Gd3+ . Unique features that make the PLGA an appropriate carrier include biocompatibility, biodegradability, more water solubility, availability of raw materials and low cost [17-19].

MATERIALS AND METHODS

All chemical reagents were of analytical grade and used without further purification. PLGA, tryptophan benzyl ester, N,N′-Dicyclohexylcarbodiimide (DCC) and MTT were purchase from Sigma Aldrich. An infrared spectrum was measured on Perkin Elmer Spectrum BX-II spectrometer. 1H-NMR spectra was measured in DMSO-d6 by a Bruker 500 MHz instrument. Atomic force microscopy (AFM) analyses were performed by JPK Nanowizard II. Dynamic light scattering (DLS) was measured using Malvern nano-zs.MCF-7 cell line was obtained from the Pasteur Institute (Tehran, Iran). ICP-MS was performed by Elan 6100 DRC-e, Perkin-Elmer. Scanning Electron Microscopic (SEM) images was obtained via Hitachi 4160 (Tokyo, Japan).

Plga conjugation with tryptophan

PLGA was purchased from Sigma Aldrich. Then, PLGA-tryptophan (nano-conjugate) was prepared by the following method. In brief, 0.05 g PLGA was dissolved in DMSO (pH=7) to react with L- tryptophan benzyl ester (0.17 g) in presence of N,N′-Dicyclohexylcarbodiimide (DCC; 0.74 g) and constantly stirred for 4 days at room temperature to react completely. The protecting group was removed by Pd(OH)2-C in 1:1 methanol:t-butanol under a hydrogen atmosphere. Dialysis was done to purify the reaction’s product. Finally, Gd3+ loading on the nano-conjugate prepared by mixture of 10 mmole GdCl3 to 1 μmole of nano-conjugate at room temperature and the reaction allowed to stir at pH=7 for 2 hr.

Scheme 1. The preparation routes of PLGA-tryptophan conjugates

Determination of Gd3+ -Trp-PLGA

SEM, AFM, Zeta- sizer, HNMR and FTIR analysis were carried out to confirm PLG Conjugation with Tryptophan. Also, FTIR was used to characterize the synthesis of Gd3+ -Trp-PLGA.

Determination of the amount of tryptophan in the conjugate

Tryptophan concentration was measured by standard curve. A standard stock of tryptophan:DMF (20mg/100mL) was prepared and a standard curve is created by testing a 200, 400, 600, 800, 1000, and 1200μL of standard solution in 10 mL of DMF containing 4 to 24 µg/mL (tryptophan/DMF). The absorbance was measured in 280 nm and standard curve in terms of absorbance versus concentration is drawn. For determination the amount of tryptophan in nano-conjugate, 30mg/100mL of PLGA-tryptophan/DMF diluted by 200 mL of DMF. Afterward absorbance of the 1mg/1mL of the solution was measured by UV spectrophotometer and amount of tryptophan determined.
In-vitro cellular uptake of plga-tryptophan-Gd3+-Vainductive coupled plasma-Mass spectroscopy (Icp-Ms)

ICP-MS was performed to assessments of PLGA-tryptophan-Gd3+ uptake by MCF-7 cell line. Briefly, PLGA-tryptophan-Gd3+and magnevist (200 and 400 μg/mL) were incubated with MCF-7 cells (25,000 cells/well) into six-well plates for 60 min at 37°C and 5% CO2. Subsequently, the cells were washed with 100 μL of phosphate-buffered saline and then centrifuged at 1000 rpm. The previous step repeated 3 times. Afterward, the samples (cell pellets) were diluted in 100 ml deionized water and the prepared samples were measured by ICP-MS.

In vitro apoptosis necrosis assay

An Annexin V-Propidium iodide staining kit was consumed to assess apoptosis according to the manufacturer’s instruction. HEK293 cell line (5000 cells/well) was used for the cell viability test. The cells were incubated with different amounts of conjugation and same amount of Magnevist for 48 hours with untreated cells as a positive control. Each concentration was tested in duplicate.

Relativity times

The MRI relaxation times for PLGA-tryptophan-Gd3+ was measured using 1.5 T.

Statistical analysis

Statistical data analysis was done using Prism5 and excels software (Microsoft Office 2013). For quantitative data analysis, One Way ANOVA in case of cluster comparison were applied. P <0.05 was considered statistically significant.

RESULT

Characterization Of conjugate

Schematic illustration of the chemical synthesis of PLGA-tryptophan conjugation is shown in Scheme 1. A FT-IR spectrum of the conjugate is presented in Fig 1.

The band at 1758 cm⁻¹ can be assigned as characteristic carbonyl bond. Carbon-carbon double bond can be observed in 1624 cm⁻¹. (N–H) bond is shown its absorption in 3322 cm⁻¹. (C–H) stretching bands are observed in 2930 cm⁻¹. A FT-IR spectrum of the PLGA-tryptophan-Gd3+ is shown in Fig 2. (O–H) bond is observed in 3398 cm⁻¹. Fig 3 indicates the 1H-NMR spectrum characterizes the PLGA-Tryptophan.
PLGA-tryptophan and PLGA-tryptophan-Gd3+ were determined -10.33, -6.15 (mV) and 250, 589.6 (nm) respectively by dynamic light scattering (DLS) technique are shown in Fig 4. The nano-carrier exhibits a narrow size distribution. Surface morphology investigation was characterized by AFM technique and the AFM images are presented in Fig 5(a:two dementional AFM Fig and 5-b:three dimentional Figs from PLGA-tryptophan-Gd3+). Scanning electron microscopy (SEM) was used to evaluate PLGA-tryptophan-Gd3+ morphology and size (Fig 6- a through d) in different scales from 2 µg to 50 µg.

![Fig 4. Size (A) and zeta potential (B) of PLGA-tryptophan (blue line) and PLGA-tryptophan-Gd3+ (red line)](image)

![Fig 5. Atomic force microscopy image of the PLGA-tryptophan-Gd3+](image)

Table 1. Data obtain from UV spectrophotometer to determine a concentration of tryptophan in nano-conjugate

<table>
<thead>
<tr>
<th>Test (30 mg/100 mL)</th>
<th>Abs.</th>
<th>Calculated concentration (µg/mL)</th>
<th>Assay (W/W %)</th>
<th>Assay (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2866</td>
<td>10.50</td>
<td>3.50</td>
<td>35</td>
</tr>
</tbody>
</table>

**Determination of the amount of tryptophan in conjugate**

Standard curve for tryptophan to assessing the amount of tryptophan in PLGA-tryptophan conjugate is shown in Fig 7 and the concentration of tryptophan in conjugate is presented in table 1.

![Fig 7. Standard curve of tryptophan in different concentrations at 280 nm](image)

**Cellular uptake of PLGA-tryptophan-Gd3+**

In-vitro cellular uptake of PLGA-tryptophan-Gd3+ at 60 min by MCF-7 cell line was found to be 58% by spectrophotometry compared to...
magnevist which is 9%. Data is shown in Fig 8.

**DISCUSSION**

Synthetic nano-carriers are an emerging diverse tool for development of novel biomedical imaging agents. Various surface chemistries and large surface area compared to their volume suggest their potential as probe for biomedical applications. Although, many types of nano-carriers were investigated, polymeric nano-structure has drawn more attention, thanks to their properties including easy synthesis or natural source preparation. Among all polymers, PLGA has several advantages including biodegradability, biocompatibility and human use permission [20-22]. By delivering nutrients such as, amino acids to an actively growing tumor site, useful imaging can be obtained [23-25].

Principally, tryptophan has been considered more valuable because of the cell metabolism association [26]. Therefore, this study was focused on synthesizing nanobiomolecular agent by considering its potential to load the Gd3+, biocompatibility and biodegradability.

The first claim of this study there to prepare the PLGA-tryptophan conjugates. For this purpose, PLGA was prepared by Sigma Aldrich and tryptophan conjugation as described in method section. The successful synthesis of the PLGA-tryptophan was confirmed by FT-IR and 1HNMR spectrums.

The existence of peaks at the region of 1.5-5 ppm indicates the aliphatic section and the peak at 6.5-7.5 ppm is related to the aromatic moiety which approves the presence of tryptophan. Moreover, apperance of carbon-carbon double
bond in 1624 cm\(^{-1}\) and (C-H) bending bands in 500-600 cm\(^{-1}\) in FT-IR spectrum confirmed the presence of tryptophan. Using the standard curve and UV spectrophotometer at 280 nm, amount of tryptophan in PLGA nano-structure found to be 35 µg/mg.

The second claim is exploring the PLGA-tryptophan Gd\(^{3+}\) loading potential and producing enough relaxivity. FT-IR spectrum of the PLGA-tryptophan-Gd\(^{3+}\) and elevation in the size and zeta potential of the nano conjugate confirm that this step is correctly done. The morphology of PLGA-Trp-Gd\(^{3+}\) nanostructures were obtained in different scales by scanning electron microscopy (SEM). The PLGA-Trp-Gd\(^{3+}\) nanostructures have a similar rod morphology. In Fig 6(a), the light contrast can be related to the gadolinium-loaded PLGA-Trp structures. The as-prepared particles size of the PLGA-Trp-Gd\(^{3+}\) nanostructures were approximately 600-700 nm. Based on the SEM images and DLS, PLGA-Trp-Gd\(^{3+}\) was successfully synthesized for MRI imaging of tumor. Relaxivities were also measured and the result showed that PLGA-tryptophan-Gd\(^{3+}\) is a good T1-weighted contrast agent. This improved of MRI relaxivity related to the availability of water molecules at paramagnetic centers. MTT (in vitro cytotoxicity) assay disclosed that nontoxic concentration up to 400µg/ml was obtained following interaction of PLGA-tryptophan-Gd\(^{3+}\) for 48 h with MCF-7 cell line. In contrast to magnevist, the nano-conjugates showed noteworthy enhancement in the mitochondrial activity of the cells. This means that PLGA-tryptophan-Gd\(^{3+}\) was less toxic compared to magnevist. Interestingly, in vitro cellular uptake by ICP-MS directly shows that 58% amount of PLGA-tryptophan-Gd\(^{3+}\) could be absorbed into the cells. In contrast to cellular uptake of magnevist (9%), PLGA-tryptophan-Gd\(^{3+}\) had more cellular uptake due to the nanostructure of the PLGA-tryptophan-Gd\(^{3+}\). More studies need to be done to understand the whole characteristics of the nano-conjugate. However, these advantages include biocompatibility and biodegradability, no cytotoxicity, high relaxivity, good cellular uptake compared to magnevist are very interesting for the PLGA-tryptophan-Gd\(^{3+}\) as a MRI imaging agent.

**CONCLUSION**

The results obtained in this study represent that PLGA-tryptophan-Gd\(^{3+}\) nano-probe successfully synthesized with proper characteristics and seems to be a good nano-probe as a MRI imaging agent. PLGA was selected because of its biodegradability, biocompatibility, good water solubility and low toxicity, potentially an appropriate candidate for using as a MRI imaging agent.

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