Functionalization of ZnO nanoparticles by 3-mercaptopropionic acid for aqueous curcumin delivery: Synthesis, characterization, and anticancer assessment

Seyed-Behnam Ghaffari a, Mohammad-Hossein Sarrafzadeh a,*, Zahra Fakhrouieian a, Shadab Shahriari b, M. Reza Khorramizadeh b,c,⁎, Zahra Fakhrouieian a, Shadab Shahriari b, M. Reza Khorramizadeh b,c,⁎⁎

a School of Chemical Engineering, College of Engineering, University of Tehran, Tehran, Iran
b Endocrinology and Metabolism Research Center, Endocrinology and Metabolism Clinical Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran
c Biosensor Research Center, Endocrinology and Metabolism Molecular–Cellular Sciences Institute, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Inherent biocompatibility and stability of zinc oxide nanoparticles (ZnO-NPs) and their biomedical potentials make them an emerging candidate for drug delivery. The aim of this study was to develop and assess a simple procedure for surface functionalization of ZnO-NPs by 3-mercaptopropionic acid (MPA) for water-soluble curcumin delivery. Carboxyl-terminated ZnO nanoparticles were successfully made using ZnCl2 and NaOH in the presence of MPA. The functional groups were activated by 1,1'-carbonyldiimidazole (CDI) and the curcumin bonding was carried out at room temperature for 24 h. The core-shell nanocomposite had a significant better solubility versus free curcumin, as characterized by XRD, FTIR, UV–Vis spectrophotometry, DLS, and TEM, p < 0.005. In addition, MTT cytotoxicity assessment on MDA-MB-231 breast cancer cells revealed a drop of IC50 values from 5 μg/mL to 3.3 μg/mL for free curcumin and ZnO-MPA-curcumin complex, respectively. This result showed an augmented cancer-inhibitory effect of nanocomposite complex. In conclusion, the presented improved solubility and elevated functionality of novel ZnO-MPA-curcumin nanoformula is promising, and could be considered for new therapeutic endeavors.

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1. Introduction

The remarkable high surface area, appropriate biocompatibility, low toxicity and chemical stability characteristics of zinc oxide nanoparticles (ZnO-NPs) have attracted increasing recent biomedical investigations. Bearing unique physicochemical properties, i.e. piezoelectric, catalytic and optic features [1], ZnO-NPs has been studied for biosensing [2], imaging [3] and antibacterial [4] applications, as well.

Hanley and co-workers have initially indicated a selective capability of ZnO nanoparticles to destruct cancerous T cells, escaping normal cells [5]. More reports on inherent cancer invading functions, bioimaging, and photodynamic therapy (PDT) properties of ZnO nanostructures have been cited ever since [6–10].

Identification and manipulation of the parameters by which ZnO-NPs contribute destruction of tumor cells maintain customizing and improving the potential biomedical applications of these nanoparticles. Particle size, surface charge, shape, solubility and surface chemistry are main physicochemical characteristics that have been showed to play critical role in determining biological behaviors of nanoparticles [11–13].

It has been reported that ZnO-NPs can readily agglomerate, owing to their high surface energy [14]. Therefore, having an applicable strategy to control ZnO-NPs agglomeration, e.g. surfactant coating or capping, is crucial to encounter biofluids. In addition, for such biological applications as sensing or drug delivery, the surface of ZnO-NPs should be functionalized to preserve water solubility and probe/drugs bioconjugate attachment. Although, bulkier form of ZnO particles have been recognized as generally recognized as safe (GRAS) material by the FDA and many researchers have indicated the low toxicity of their nano-scale form against normal cells, coating of nanoparticles is also an appropriate method for reducing unwanted toxicity and improving biocompatibility of nanoparticles [8]. To date, a number of capping agents or polymeric compounds have been employed to modify and functionalize ZnO nanoparticles: 3-aminopropyltriethoxysilane (APTS) [15],

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monooethanolamine (MEA) [16], mercaptoundecanoic acid (MUA) [17], mercaptacetic acid (MAA) [18] and fatty acid [19], respectively.

Curcumin, an active ingredient of turmeric, has been extensively investigated in the treatment of various illnesses, including cancer, cardiovascular disease, inflammatory bowel, wound healing, Alzheimer’s disease, rheumatoid arthritis, and diabetes [20]. However, low water solubility and bioavailability, rapid metabolization, and poor chemical stability have rendered utilization of curcumin limited in clinical trials [21]. Curcumin was reported undetectable when its daily oral administration was < 8 g [22].

To overcome these problems, various types of curcumin nanoformulations, usually referred to as curcumin-loaded organic systems have been developed: solid lipid nanoparticles, micelles, liposomes, nanogels, cyclodextrins, and dendrimers [23]. In line with the increasing applications of metal and metal oxide nanoparticles as nanocarriers, researchers have developed nanoformulations in which curcumin molecules are bound to gold [24], iron oxide [25], silica [26], and recently, zinc oxide [27] nanoparticles. These systems demonstrate advantages as easily tailored for favorable therapeutic purposes.

In this study, a novel ZnO-NPs functionalization procedure, using 3-mercaptopropionic acid, was developed for curcumin conjugation in a water-soluble delivery system. This nanoformulation can enhance curcumin solubility and stability. The enhanced bioavailability, together with inherent preferential tumor toxicity of ZnO-NPs, eventually improves therapeutic applicability of curcumin. Several characterization techniques were performed to analyze the physicochemical properties of this engineered core-shell nanocomposite and its anticancer activity on MDA-MB-231 breast cancer cell lines has been evaluated by MTT assay.

2. Material and methods

2.1. Chemicals, cell line and initial cell culture

Zinc chloride, Sodium hydroxide, 3-mercaptopropionic acid, 1,1′-carbonyldiimidazole (CDI) and curcumin were purchased from Merck. Dried dimethylsulphoxide (DMSO) and ethanol were obtained from Ameretat Shimi, Iran. MDA-MB-231 breast cancer cell line (German Collection of Microorganisms and Cell Cultures, DSMZ, Braunschweig, Germany) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin in of attached curcumin was calculated using the formula: Amount of curcumin conjugated = Total amount of curcumin used − the amount of curcumin in the supernatant.

2.4. Characterization of products

The phase characterization of as-synthesized materials was obtained by an X-ray diffractometer (XRD; Siemens D-500). The analysis was done in the 2θ range from 10 to 90° using copper Kα (λ = 1.54056 Å) radiation. The crystallite sizes of the products were calculated by Scherer’s formula [28].

The particle size and morphology of the products was investigated using a transmission electron microscope (TEM; Zeiss-EM10C-100 KV) and a field-emission scanning electron microscope equipped with an EDAX energy dispersive X-ray analyzer (SEM; Zeiss-SIGMA VP-500).

The hydrodynamic size and zeta potential of the prepared particles were measured with Dynamic light scattering (DLS; ZetaPlus, Brookhaven Instruments). The procedure of DLS samples preparation included a dispersion of nanoparticles in distilled water (1 mg/mL) and subsequently sonication for 5 min (bath sonication). To investigate the effect of serum on the hydrodynamic size of the conjugate product, the complex dispersed first in DMED + 10% PBS (1 mg/mL) and stirred for 3 h and then re-dispersed in water.

Fourier Transform Infrared (FTIR) spectra were recorded with Perkin Elmer FT-R Spectrometer, using KBir pellets, in the region of 400–4000 cm⁻¹. Ultraviolet–visible (UV–Vis) spectra were recorded by OPTIZEN 2120UV plus spectrophotometer in the range of 200–600 nm.

2.5. Estimation of curcumin content in the conjugate product

Standard calibration curve of curcumin in DMSO was obtained from the absorption intensity by UV–Vis spectroscopy at 430 nm. The concentration of curcumin in the conjugate product was estimated by measuring the present of free curcumin (non-attached curcumin) in the supernatant of synthesis procedure of ZnO-MAA-curcumin. The amount of attached curcumin was calculated using the formula:

Amount of curcumin conjugated = Total amount of curcumin used − the amount of curcumin in the supernatant.

2.6. Sedimentation study

The sedimentation rate of ZnO-MAA-curcumin nanoparticles was investigated by tracking the optical absorbance at 400 nm as a function of time, using UV–Vis spectrophotometry.

2.7. In vitro cytotoxicity assay

An optimized MTT reagent-based colorimetric assay was carried out to assess possible cytotoxic effects of native curcumin, ZnO-MAA NPs and ZnO-MAA-curcumin reagents, respectively [29]. Briefly, MDA-MB-231 breast cancer cells were plated into 96 well plates at a density of 10,000 cells/well in 200 μL complete culture medium and allowed to attach overnight. Cells in the exponential phase of growth, were treated with desired concentrations of the above-named treatment reagents for 48 h. Dispersion of the samples in cell culture was done using a bath sonicator for 5 min. After treatment, cells were washed twice with PBS and then 20 μL of MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich) dissolved in PBS (4 mg/mL) was added directly to all the wells, and the plates were incubated for 3 h at 37 °C. After removal of the supernatant, the formazan crystals that formed were dissolved by addition of 60 μL DMSO per well. The color intensity resulting from the formation of soluble formazan product was measured at 570 nm with 660 nm set as the reference wavelength using a microplate reader (Anthos, Austria). This absorption reading is directly proportional to the number of live cells in the culture.
The percentage of viability was presented as the percentage of the absorption of treated cells to the absorption of untreated cells as the negative control. The maximum DMSO percentage as the solvent of curcumin in cell culture was 0.5% (v/v). DMSO 0.5% showed an absorbance value similar to control indicating that DMSO 0.5% have no effect on the cells. Data presented are from three replicates.

3. Results and discussion

3.1. ZnO functionalization by 3-mercaptopropionic acid

Zinc oxide nanoparticles, either alone or loaded with biological probes, are considered as novel compounds for cancer treatment and biosensing researches. One applicable approach to enhance biomedical properties, could be conjugation of monodispersed ZnO-NPs with similar-effect biomolecules. Various methods have been developed to functionalize ZnO-NPs and improve their water solubility and size stability. In the present study, a simple chemical approach was designed to produce functionalized zinc oxide nanoparticles from zinc chloride solution using MPA as a capping agent containing a carboxyl group. This functional group can provide improved NPs aqueous-solubility and enhanced linkage to biological molecules which usually contain different functional groups like amide or hydroxyl moieties.

The formation mechanism of zinc oxide crystals in the chloride solutions was comprehensively discussed elsewhere [30]. To verify formation of ZnO phase and study the influence of the surface functionalization on the crystallinity of ZnO-NPs, XRD analysis was carried out. Fig. 1 shows the XRD pattern of this products. All the diffraction peaks in the diffraction patterns of the powders obtained from ZnO, ZnO-MPA synthesis procedures showed in accordance with the JCPDS file of hexagonal wurtzite zinc oxide (JCPDS #36-1451). Since no significant difference has been seen between the two patterns, it can be concluded that the surface modification did not change the crystalline structure of the zinc oxide nanoparticles. The crystallite size of zinc oxide tends to be smaller in the presence of MPA molecules. This value for ZnO and ZnO-MPA particles were 62 nm and 23 nm, respectively.

FTIR spectroscopy was used to investigate the incorporation of MPA to the surface of ZnO-NPs. Fig. 2 displays FTIR spectra of as-prepared powders along with free curcumin. FTIR of all samples clearly showed a broad peak between 3600 and 3050 cm⁻¹ and intense peaks between 420 and 500 cm⁻¹ reflecting the presence of hydroxyl groups and Zn—O bonds, respectively. The peak at 1398 cm⁻¹ in ZnO and ZnO-MPA is related to the C—OH bonding. This peak was shifted to 1411 cm⁻¹ in the conjugate product. The sharp peak at 1569 cm⁻¹ in the spectrum of ZnO-MPA implied the presence of –COOH groups on the surface of the modified ZnO-NPs [14]. Two chemical procedures are probably involved in the formation of chemical bonds between zinc oxide and the MPA molecules. The First procedure is dehydration reaction between carboxyl groups in MPA and hydroxyl groups at the surface of ZnO crystals. Secondly, is the replacement of the oxygen

![Fig. 1. XRD patterns of ZnO, ZnO-MPA, and ZnO-MPA-curcumin powders.](image1)

![Fig. 2. FTIR spectra of ZnO, ZnO-MPA, ZnO-MPA-curcumin and free curcumin.](image2)
atoms in ZnO crystals by S atoms of MPA molecules [17]. The absence of a peak at 2550 cm$^{-1}$ related to SH bond most likely indicates the formation of ZnO-MPA complex by the substitution reaction.

Fig. 3 shows typical TEM and SEM images and the corresponding EDS spectra of ZnO-MPA particles. Based on both microscopic images, the particles have semi-spherical morphology. The core/shell structure of the particles can also be seen in the TEM image where the core and the outer layer in particles are clearly distinguishable. Based on the TEM images of > 100 particles, the average diameter size of ZnO-MPA particles was about 38 nm. The EDS characterization revealed proportions of 61.6% zinc, 17% oxygen, 20.7% carbon, and 0.8% sulfur and indicated the presence of MPA elements in the complex.

Another technique to approve the binding of MPA to ZnO nanocrystals and to verify the presence of carboxyl groups is to compare the aqueous zeta potential of ZnO-MPA with bare ZnO particles. The point of zero charge (PZC) of ZnO is 8.4–10.3. This indicates that the charge of ZnO particles is positive in water and nutrient pH buffers. As compared with the zeta potential of bare ZnO particles (+12 mV), the surface charge of the modified ZnO nanoparticles (calculated by DLS) was −31 mV that could be associated with the negative charge of the carboxyl groups in water (Table 1). The calculated charge-differences is indicative that MPA has been bonded to the zinc oxide nanoparticles surface. The strong negative charge of the modified nanoparticles caused a reduction of the agglomeration of particles. Accordingly, the hydrodynamic size of nanoparticles decreased from 431 nm to 40 nm. This value is very close to the mean diameter size obtained by TEM images.

The UV–Visible light absorption spectra of ZnO particles and MPA-capped ZnO-NPs are illustrated in Fig. 4. ZnO and ZnO-MPA NPs exhibited their characteristic strong absorption bands corresponding to bend-edge absorption at 354 and 342 nm, respectively. This blue shift of the absorption edge in ZnO-MPA NPs compared with non-modified ZnO could be attributed to a quantum confinement effect, probably due to the smaller size and less agglomeration of the modified ZnO-NPs.

### 3.2. Curcumin bonding to MPA capped ZnO-NPs

The direct conversion of a carboxylic acid to an ester with hydroxyl groups on the curcumin molecule is a very complicated process. In contrast, formation of ester moiety would be much easier, if the carboxylic groups are first activated. 1,1′-carbonyldiimidazole (CDI) is a highly efficient activating-agent for carboxylic acids. Moreover, fewer side reactions are associated with CDI, as compared to other alternative molecules like dicyclohexylcarbodiimide (DCC). The activation procedure was performed in DMSO, as DMSO is a suitable solvent for curcumin. During the process, a reactive intermediate, N-acylimidazole, is generated which can then react with hydroxyl groups in curcumin to form ester linkages [31].
FTIR spectrum of curcumin showed peaks at: i) 1628 cm$^{-1}$, indicating C–C symmetric aromatic ring stretching; ii) 1508 cm$^{-1}$ contributed to C–O of benzene ring; iii) 1427 cm$^{-1}$ associated with C–H bending vibration; iv) 1279 cm$^{-1}$ represents aromatic C=O stretching vibrations; and v) 961 cm$^{-1}$ related to C–O–C stretching vibrations (Fig. 2). FTIR spectrum of ZnO-MPA-curcumin conjugate demonstrated peaks related to ZnO-MPA. In addition, curcumin-related main peaks, especially the peak at 1490 cm$^{-1}$, is assigned to C=O of aromatic rings of curcumin. The sharp peak at 1090 cm$^{-1}$ clearly indicated the esterification of the carboxyl groups and the formation of the ester linkage (C–O–C). These results confirm the covalent attachment of curcumin to the surface of ZnO-MPA NPs.

XRD pattern of the conjugate product observed in Fig. 1 has no obvious differences with the pattern of ZnO-MPA NPs. This indicates the amorphous nature of curcumin, bonded to the surface of modified ZnO-NPs. Crystallite size of ZnO in the conjugate product was 55 nm.

In the present study, the light absorption behavior of free curcumin has been analyzed in methanol. Curcumin has an intense absorption between the wavelength of 340–535 nm, depending on the solvent characteristic and its structural modification [32]. According to Fig. 4, the spectrum of curcumin showed a strong maximum at 430 nm. The spectrum of ZnO-MPA-curcumin exhibited two absorption maximums at 348 and 425 nm, respectively. The presence of the peak at 425 nm, related to the adsorption behavior of curcumin, clearly indicated that the drug adsorbed well on the surface of ZnO-MPA nanoparticles. The mere 5 nm blue shift in the maximum wavelength peak was derived from the reaction of hydroxyl groups with the activated carboxyl groups and the formation of chemical bonds between MPA and curcumin. The active concentration of curcumin in the complex was estimated about 130 μg per mg of the conjugate (about 13% w:w of the complex).

Shape and size particle of the conjugate product were studied by SEM-EDS, TEM, and dynamic light scattering (DLS). The EDS characterization of the particles (Fig. 5a) revealed proportions of 46% zinc, 18.4% oxygen, 35.2% carbon, and 0.4% sulfur. A relatively higher proportion of carbon was indicative of curcumin molecules bindings. The SEM image shows the semi-spherical morphology of particles (Fig. 5b). Core-shell and a multi-layer structure of the ZnO-MPA-curcumin formulation and its hypothetical schematic were demonstrated in TEM images (Fig. 5c, d and e) where ZnO is the core and MPA and curcumin molecules are positioned as the second and third layers, respectively. The average diameter size of the core was about 74 nm and the mean diameter of the total complex was about 121 nm. Therefore, this core-shell structure can be concluded as a nanocomposite. The size of ZnO-MPA area is larger, as compared with TEM image in Fig. 3. This size discrepancy derived from probable agglomeration of ZnO-MPA particles in the activation step.

Shape and size particle of the conjugate product in body fluids is very important for drug delivery purposes. The behavior of the particles was therefore investigated in water and in serum-containing media (simulating blood plasma condition). As shown in Table 1, the hydrodynamic size of the conjugate product, when dispersed in de-ionized water, was

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hydrodynamic size (nm)</th>
<th>Polydispersity index</th>
<th>Zeta potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnO</td>
<td>431</td>
<td>0.35</td>
<td>+12</td>
</tr>
<tr>
<td>ZnO-MPA</td>
<td>40 nm</td>
<td>0.12</td>
<td>−31</td>
</tr>
<tr>
<td>ZnO-MPA-curcumin</td>
<td>904 nm</td>
<td>0.243</td>
<td>−15</td>
</tr>
<tr>
<td>ZnO-MPA-curcumin</td>
<td>157 nm in DMEM + 10% FBS</td>
<td>0.176</td>
<td>−</td>
</tr>
</tbody>
</table>

Table 1 Hydrodynamic sizes and zeta potentials of the samples.

**Fig. 4.** UV–Visible spectra of (a) ZnO and ZnO-MPA NPs and (b) ZnO-MPA-curcumin and free curcumin.
approximately 904 nm, depicting possible aggregated curcumin-coated ZnO-MPA nanoparticles. This aggregation could be resulted from hydrophobic nature of curcumin and strong reduction of the zeta potential of the particles (shifting from $-15$ mV to $-31$ mV). The agglomeration of the conjugate particles was decreased clearly to 157 nm when they were initially suspended in cell culture medium supplemented with serum (DMEM + 10% FBS) and then, re-dispersed in water. The amelioration of agglomerated-NPs problem could presumably be due to protein adsorption on the surfaces of particles [33]. This hydrodynamic size (157 nm) has been reported as an effective size for the purpose of passive delivery to tumor cells (<200 nm but >10 nm), as well [34].

Furthermore, particle sedimentation rate of the conjugate product showed dependence on the suspending media. Fig. 6 represented the sedimentation rate of ZnO-MPA-curcumin nanocomposite in de-ionized water and in the cell culture medium containing 10% serum. The particles in water displayed a faster deposition rate than those in the...

Fig. 5. (a) EDS spectra and (b) SEM image of the conjugate product. (c) and (d) TEM images of ZnO-MPA-curcumin nanocomposite and (e) its hypothetical schematic.
cell culture medium. Almost 40% of the particles precipitated within 24 h in water. Conversely, free suspended curcumin precipitated immediately in water. However, the conjugate particles could extremely disperse better. The sedimentation rate was significantly slower in the cell culture medium with serum (only 8% of the particles deposited in the same time period). Therefore, pre-dispersion of the conjugate particles in serum-containing solution can be a stabilizing method and a suitable technique for biological studies.

3.3. Anticancer potential of the conjugate product

Conjugation of anticancer drug molecules may alter their functionality. It is therefore imperative to re-assess the anticancer properties of the complexed molecules after the conjugation process. Cytotoxicity tests are employed as the bottom line analyses, accordingly. MTT assay revealed curcumin, ZnO-MPA nanoparticles, and the conjugate product cytotoxic effects on MDA-MB-231 breast cancer cell model in a dose-dependent manner, respectively (Fig. 7).

When cells were treated with curcumin, the viability of the cancer cells decreased sharply in a dose-dependent manner. A similar pattern could be seen with ZnO-MPA and ZnO-MPA-curcumin at all tested concentrations. The half maximal inhibitory concentration (IC50: 50% cell growth inhibitory concentration) was calculated as 5 μg/mL (curcumin), 38 μg/mL (ZnO-MPA), and 25 μg/mL (ZnO-MPA-curcumin), respectively. IC50 value of the conjugated molecules, calculated from curcumin concentration base curve, was about 3.3 μg/mL which is higher than that of free curcumin. This interesting finding revealed the augmented cancer inhibitory effects of conjugated molecules, as compared to free curcumin. In other words, anticancer property of ZnO nanoparticles has been preserved during the nanoformulation and conjugation methodology presented in this study.

Fig. 7. Viability of MDA-MB-231 cells (MTT assay) after 48 h exposure to (a) free curcumin (b) ZnO-MPA NPs (c) ZnO-MPA-curcumin nanocomposite and (d) ZnO-MPA-curcumin nanocomposite based on the concentration of curcumin in the complex. Data represented as the means ± SD of three identical experiments derived from three replicate.
4. Conclusions

In this study, ZnO-NPs were successfully functionalized via a novel chemical procedure using 3-mercaptopropionic acid (MPA). This moiety was covalently conjugated to curcumin. The synthesized conjugate product was then characterized, and its cytotoxicity was assessed using MDA-MB-231 breast cancer cell model. Considering the inherent anticancer effect of zinc oxide and the capability of functionalization to attach drugs makes ZnO-NPs favorable for drug delivery applications. The MPA functionalization procedure presented in this study might be amply customized to form targeted drug delivery endeavors. Curcumin, as a natural molecule with several therapeutic potentials, primarily suffers from low solubility and bioavailability in its free molecule form; was covalently conjugated to the surface of as-synthesized ZnO-MPA NPs. Physicochemical properties of the nanocomposite were evaluated using several techniques. Our results indicated that the nanocomposite had a core-shell structure and can be dispersed well in cell culture medium in the presence of serum. MTT assay indicated that not only the chemical binding of curcumin molecules to ZnO NPs did not reduce the anticancer activity of curcumin, but the conjugate product had also higher cytotoxicity against the tumor cells. The conjugate product could bear several potentials for cancer treatment such as chemotherapy, photodynamic therapy (PDT) and imaging. However, its commercialization needs a series of in-vitro and in-vivo analyses that are presently missing.

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