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Biocompatible Luminescent Nanosized Curcumin: Verified Parameters Affecting Stability and Bioavailability

Research Article

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Abstract

Purpose: Curcumin one of the herbal compounds that are used as an antitumor, antioxidant, and anti-inflammatory agent. However, curcumin applications are limited due to its poor chemical stability and low water-solubility. On the contrary, the enhanced solubility of nanocurcumin improves its bioavailability and cellular uptake. The present study aims to verify a simple, achievable, and reproducible method for preparation of biocompatible nanocurcumin and to optimize the different parameters included in this method.
Methods: In our study, we used solvent anti-solvent precipitation method to synthesis curcumin nanoparticles. For biocompatibility, we screened different organic solvents to verify the best safe one with the highest solubility effect to curcumin. Furthermore, we tested the effect of adding stabilizersto enhance the stability and yielding capacity of the nanocurcumin. Thereafter, the stabilized nanosample was tested for water-solubility and luminescence.
Results: Acetone-dissolved nanocurcumin gave clear amber yellow nanosuspension without any precipitate and with an acceptable concentration of nanosuspension. With limiting the stirring rate and time to the minimum, the nanosample was monodispersed. Furthermore, stabilized nanocurcumin revealed the highest solubility and luminescence.
Conclusion: The prompt fluorescent property of the soluble nanosized curcumin would qualify this herbal nano-candidate to be utilized as a theranostic agent.

Keywords: Nanocurcumin; Solvent Anti-Solvent; Water-Solubility; Luminescence; Theranostic.

Introduction

Nanotechnology has entered the field of dentistry, resulting in a magnitude evolution in dental materials and innovations in oral health-related diagnostic and therapeutic methods. Thematerials at a nanometric scale (10-9 cm) has unique physical, optical, and electrical properties differ from their bulk form [1]. Inorganic nanoparticles have been frequently investigated in the diagnosis and treatment of oral cancer [2-4]. However, due to the reported toxicity of metallic nanoparticles, biocompatible natural polymeric nanoparticles have gained attention.Moreover, there is an increasing demand for a safe nano-candidate that could be used dually as theranostic agent [5].

Curcumin -a member of the ginger family- is a natural polyphenol widely used asan herbal supplement. Biomedically, curcumin is widely used as an anti-inflammatory and antimicrobial agent due to its potent antioxidative activity. Moreover, curcumin exhibits a unique anticancer activity through the induction of apoptosis, inhibition of proliferation, and prevention of invasion, without

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delirious effect on the adjacent healthy cells [6-8].

Our team is interested in using herbal medicine, including curcumin, in combating oral squamous cell carcinoma in vivo. However, curcumin has shown limited bioavailability, clinical efficacy, and low cellular uptake [9]. The poor water-solubility of the curcuminas well as low chemical stability, consider as major obstacles hindering its biological use. The curcumin molecule, due to its hydrophobicity, tends to bind to the phospholipid of the cell membrane by hydrogen bond, resulting in low availability of curcumin inside the cytoplasm [10, 11]. Despite curcumin is soluble in different organic solvents, the reported toxicity of these solvents limits their biological uses.Therefore, curcumin water-solubility is still the target for biocompatibility and safety purposes. One of the most promising solutions for curcumin hydrophobicity is synthesizing curcumin at nano-size [12]. Moreover, the water-soluble nano-sized curcumin exhibits characteristicluminescent optical properties that can be safely utilized in diagnostic nanomedicine [13].

The solvent anti-solvent precipitation method is one of thepromising bottom-up approaches for nanocurcumin preparation. Curcumin nanoparticles precipitate as a result of the difference of saturation caused by mixing the solution and the anti-solvent. The way for producing nanoparticles by solvent anti-solvent precipitation is to create conditions that enhance very rapid particle formation without or little particle growth. The main advantages of this approach are the simplicity, cost-effectiveness, and theeasiness to scale-up the nanoparticles ideally [14, 15]. However, the solvent anti-solvent precipitation method has many variables that constrain its reproducibility and affect the sample stability. For example, there are different organic solvents, where curcumin shows different solubility rates. Furthermore, there are reservations on some organic solvents due to their toxicity and environmental aspects concerning their recycling and separation from the anti-solvent [15].

Our study, therefore, aims to optimize a simple, achievable, and reproducible method for the preparation of curcumin nanoparticles. We screened different organic solvents and stabilizers to reach the most potent biocompatible one, with superior yielding capacity and stability. We concluded that the smallest polyvinyl pyrrolidone coated nanocurcumin revealed uppermost stability, bioavailability, and auto-fluorescence.

Materials and Methods

Material

Curcumin powder was purchased from Alpha Chemika (Mumbai, India). The coating agents; polyvinylpyrrolidone (PVP; Mw 40,000) and polyethylene glycol (PEG; Mw 6000), as well ascell staining chemicals; Hoechst (H6024-10ML) and fluorescein isothiocyanate (FITC) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Loughborough, UK). Acetone and all other reagents were of analytical grade and used as received. The used deionized water (DIH_2O) used to was ultra-purified from Millipore Milli-Q system (resistivity ~80 MΩ cm).

Preparation of curcumin nanoparticles

We prepared the curcumin nanoparticles by the solvent anti-solvent precipitation technique toinvestigate the effect of different parameters on size, dispersity, yielding capacity, and stability of the synthesized curcumin nanoparticles.

First, we screened the solubility rate of curcumin in different organic solvents by dissolving 10mg of curcumin powder in 1 ml of dichloromethane, ethanol, DMSO, and acetone. Then, the curcumin solutions were added slowly dropwise to 15ml DIH_2O under stirring at room temperature (25°C), using different stirring rates (500, 800, and 1000rpm) and times (1 and 5 min) [16].

After that, we assessed the effect of different stabilizers with different ratios by adding 0.5% and 1%w/v of PVP and PEG to DI-H2O before adding the curcumin solution.Finally, the nanosuspensions werefreeze-dried by (BachiLyovapor L-200,Switzerland) [17].

Characterization of curcumin nanoparticles

The preliminary detection of synthesized curcumin nanoparticles was carried out by UV–visible spectrophotometer (Nanodrop, DeNovix, DS-11 FX+, US)with scanning theabsorbance spectra in the range of 200–800nm [18].

The average particle size, polydispersity index (PDI), and particle charge of curcumin nanoparticles were performed by the dynamic light scattering technique using Zeta-seizer (Nano ZS, Malvern Instruments, Worcestershire, UK), with adilution ratio of 1:6. All measurements were carried out in triplicate and performed at room temperature [16].

The morphology and size of the synthesized curcumin nanoparticles were characterized by transmission electron microscope(TEM; JOEL, JSM-6360LA, JAPAN) [19].

Fourier transforms infrared spectroscopy (FTIR)analysis of curcumin, stabilizer, and nanocurcumin was performed using an FTIR spectrometer (PerkinElmer Inc, Shelton, CT, USA).A spectrum for each sample was recorded within therange of 4000-500 cm^{-1} [17].

Solubility test

For assessing water-solubility of the nanocurcumin versus curcumin powder, we dissolved 10 mg oflyophilized nanocurcumin powder in 1 ml DIH_2O, with comparing the solubility of an equivalent dose of curcumin powder. Furthermore, we assessed the nanocurcumin solubility in Dulbecco's Modified Eagle's Medium (DMEM) [20].

Standard curve

To reach the extinction coefficient (ε) of the stabilized nanocurcumin, we performed the standard curve, by preparing serial concentrations of (5, 4, 3, 2, and 1 mg/ml) DIH_2O-soluble nanocurcumin. For plotting the standard curve of the curcumin nanoparticles, UV–visible spectrophotometer was done to obtain the absorption of each dilution. Then the absorbance (λ) was plotted on the y-axis and concentration (c) on the x-axis to gain the equation of Beer-Lambert law ($c = \lambda/\varepsilon$) [21].

In situ and *in vitro* luminescent verification

The Alexandria University Ethics Committee approved the study and the procedures followed are under the institutional guidelines (IRBNO:00010556-IORG0008839). The photoluminescence of water-soluble nanocurcumin was recordedby spectrofluorometer (Nanodrop, DeNovix, DS-11 FX+, US) at specified excitation wavelengths;470, 525, and 635nm to determine the absorption at each wavelength. To confirm the emission wavelength of the nanocurcumin, flow cytometric analysis was made at 488nm blue laser by flow cytometry (BD Biosciences, Germany). We prepared 1ml of DIH_2O-soluble nanocurcumin of 3 mM [13].

For *in vitro* visualization of the curcumin nanoparticles, gingival fibroblast cells were seeded on cover glasses in a 6-well plate at the density of $5x10^4$ cells per well. Cells were treated with 50 µM nanocurcumin dissolved in DMEM for 4 hours at 37°C in a 5% CO_2 incubator. Cells treatment with an equivalent concentration of FITCwas used as positive control. The cells were fixed with 4% paraformaldehyde, after which they were permeabilized by Triton X (0.2%). Finally, the cells were stained by Hoechst and mounted on glass slides. We examined the cells under a confocal laser scanning microscope (Leica TCS SPE, Germany) equipped with imaging software (Leica LASX, Germany). Then, we analyzed the intensity of fluorescence morphometrically using an image analysis software (Image J; 1.52p software 32, NIH, USA) [22].

Statistical analysis

We used IBMSPSSsoftwarepackage version 19.0 (IBM Inc., Chicago IL, USA) to analyze the data. Considering normally distributed variables, one-way ANOVA and Tukey multiple range tests were conducted.Data are described as mean ± standard deviation (SD) and the significant difference is determined at P-values < 0.05.

Results

Acetone dissolved curcumin is the highly soluble solution with the highest yielding capacity of nanosuspension

In our screening of the solubility rate of curcumin in different organic solvents, curcumin powder was highly soluble in acetone and DMSO, giving clear amber yellow and deep-orange solutions, respectively. On the other hand, curcumin was less soluble in dichloromethane and ethanol. Bothsolvents gave turbid yellow-orange solutions with precipitate (Fig. 1A).

Upon addition of the curcuminsolutions dropwise to DIH_2O under stirring, the resulted nanosuspensions were variable. The DMSO revealed an immediate deep-orange precipitate, while dichloromethane and ethanol gave yellow precipitate. On the other hand, acetone gave clear amber yellow nanosuspension, without any precipitate, (Fig. 1B).

Fig. 1C shows the UV–visible spectrophotometer of acetone-dissolved nanocurcumin with a narrow smooth regular peak and specific absorbance at 419nm, which is the characteristic peak of nanocurcumin. Moreover, curcumin-acetone yielding capacity was higher than other different organic solvents. Meanwhile, nanocurcumin dissolved in different organic solvents (DMSO, dichloromethane, and ethanol) reveal irregular broad peaks without specific absorbance, reflecting non homogeneous nano-populations.

Consequently, in our next trial to optimize the time and rate of stirring, we focused on the acetone-suspended nanocurcumin.

Increasing time and rate of stirring clump the suspended curcumin nanoparticles

In our trial optimizing time and rate of stirring, the synthesized nanocurcumin particles were mono-modal dispersed, with an av-

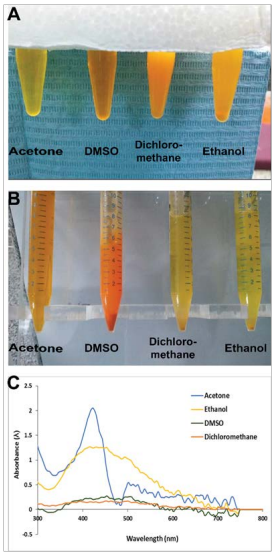

Figure 1. The physical properties of curcumin and nanocurcumin in different organic solvents and their UV-Vis spectrophotometers. (A) Curcumin is highly soluble in acetone and DMSO, while turbidity and precipitate are seen in dichloromethane and ethanol. (B) The physical properties of the corresponding nanosuspensions, where acetone-dissolved nanocurcumin is the only revealing clear nanosuspension without any precipitate. (C) The UV–Vis spectrophotometer of nanocurcumin dissolved in acetone shows a definite peak of absorbance at 419 nm with higher-yielding capacity compared to the irregular broad peaks of other organic solvents.

erage size of 160.3 ± 4.4 nm and a low PDI of 0.3 ± 0.02, on stirring power 500 rpm for 1 min. With increasing time of stirring to 5 min and rate to 800 and 1000 rpm, the average size increased significantly to 230.1 ± 11.5 nm and 242.5 ± 14.1 nm, respectively (P<0.001). Furthermore, the nanoparticles clumped and became multimodal dispersed, with significant increases of PDI to 0.5 ± 0.04 at 800 rpm and 0.4 ± 0.1 at 1000 rpm (P<0.05). Therefore, in our attempts to increase the stability of nanosuspension, we limited the stirring rate to 500 rpm and stirring time for 1 min.

The PVP-coated curcumin nanoparticles are the most stable with high solubility and biocompatibility

As a result of the nonhomogeneous distributedcurcumin nanoparticles, we used coating agents to achieve more stability and superior yielding capacity. We compared two of the most intensely used pharmaceutically polymers; PVP and PEG, at different ratios. Stabilization with 0.5% PVP on stirring (1 min and 500 rpm) gave a clear yellow solution without any precipitate. While PEG with an equivalent ratio gave a turbid yellow suspension with precipitate, (Fig. 2A).

Figure 2B shows the superior UV–visible spectrophotometer results of 0.5% PVP stabilized nanocurcumin over PEG stabilized nanoparticles at the equivalent ratio. The former revealed a narrow, smooth regular peak with high yielding capacity at the characteristic absorbance peak, of nanocurcumin at 419 nm. The coating of curcumin nanoparticles with 0.5% PVP decreased the average size significantly to 121 ± 0.03 nm (P<0.001). Furthermore, it resulted in a homogenous monodispersed nanoparticles population, with low PDI of 0.2 ± 0.02 and surface charge of -12.9 mV, indicating the stabilization of nanosample, (Fig.2C-D). Meanwhile, the PEG-coated nanocurcumin was nonhomogenous with significant increases of the size to 343.9 ± 16.1 nm and PDI to 0.4 ± 0.1, with a low surface charge to -4.26 mV (P<0.001, Fig. 2E-F).

With increasing the ratio of PVP to 1%, the retrieved nanopopulation was a nonhomogenous multimodal dispersed, with significant increases of the size to 559.5 ± 49.9 nm and PDI to 0.6 ± 0.08 (P<0.001, Fig.2 G-H). Furthermore, the surface charge decreased to -0.607 mV, indicating low stability of the nanopopulation.

The TEM analysis of PVP-stabilized nanoparticles, showed round monodispersed particles with a mean size of 33.33 ± 10.1nm, (Fig. 3A). Furthermore, the FTIR results confirmed the success of synthesizing biocompatible PVP-coated nanocurcumin.Initially, comparing the simple shift in the absorbance spectrum between curcumin powder and nanoparticles in Fig. 3B indicates minor structural changes that occur during the solvent anti-solvent technique. Moreover, the absence of any peaks for acetone confirms the biocompatibility and the total purification of the synthesized dry nanocurcumin. On the other hand, the strong peaks in the ranges of 1651,1422, and 1290 cm^{-1} appeared in the nanocurcumin, compared to the corresponding ones in pure PVP, indicates the success in stabilizing the nanocurcumin.

In testing the solubility of the synthesized nanocurcumin, the PVP-stabilized nanoparticles were highly soluble in water and DMEM without any precipitate. In contrast, the solubility test proved the poor solubility of curcumin powder in water as well as in DMEM, (Fig. 3C).

According to the analytical curve in Fig. 4D, the PVP-stabilized nanosuspension revealed the superior yielding capacity of ~36.7 mM, compared to the initial 2.7 mM of curcumin powder. Consequently, the synthesis of curcumin at nano-size would help increasing its bioavailability and cellular uptake with improving its efficiency.

The auto-fluorescence property of nanocurcumin qualifies it as a promising theranostic agent

By the spectrofluorometer, the water-soluble nanocurcumin revealed thehigh intensity of 165.351 RFU (relative fluorescence unit) at the blue excitation. After determination of the excitation-emission spectra of curcumin nanoparticles, the flow cytometry results confirmed the auto-fluorescence of a median 24.14 at the similar excitation spectrum.

Interestingly, both nanocurcumin and FITC revealed their luminescence at the blue excitation, upon confocal examination. However, DMEM-soluble nanocurcumin showedintense fluorescenceof a 1553.7-fold than the FITC (P<0.000). Moreover, the luminescent nanoparticles showed nuclear and cytoplasmic localization, where FITC localized in the cytoplasm of the gingival fibroblast (Fig. 4).

Discussion

Curcumin, as a natural compound widely used in herbal medicine, has a decreased biomedical efficiency due to its poor water-solubility. Nanotechnology has been used to overcome the problems of decreased stability and bioavailability associated with poorly soluble drugs. Wesettled on the solvent anti-solvent precipitation method, which is suitable for the synthesis of highly soluble nanocurcumin. However, this approach has many variables that directly influence the particle size, stability, and dispersity of the resulted nanopopulation.

From our screening of curcumin solubility in various organic solvents, acetone was verified as the best solvent. Biologically, acetone is safe and can be easily removed from the final formulation by evaporation due to its low boiling point 56°C [16]. Physically, curcumin is highly soluble in acetone, giving a clear yellow solution without any precipitate. Our UV–visible spectrophotometer results confirmed the synthesis of curcumin nanoparticles from acetone-dissolved curcumin solution with an absorbance peak of 419nm, following the results of Alam et al., [23] and Ghosh et al., [24].

Stirring rate and time are pivotal factors affecting sample stability, dispersity, and size. In the present study, the stirring rate at 500 rpm for 1 min was significantly better than the other increased stirring rates(800 and 1000 rpm) for 5 min. In our verification, slow stirring rate was gentle enough to complete the mixing of curcumin-acetone solution with the anti-solvent. Therefore, the particle growth retained apart from each other at a smaller size, resulting in a monodispersed nanopopulation. Meanwhile, the aggressive mixing with an increased duration resulted in clump and collisions of the curcumin nanoparticles, promoting their agglomeration and growth with wide particle size distribution [16].

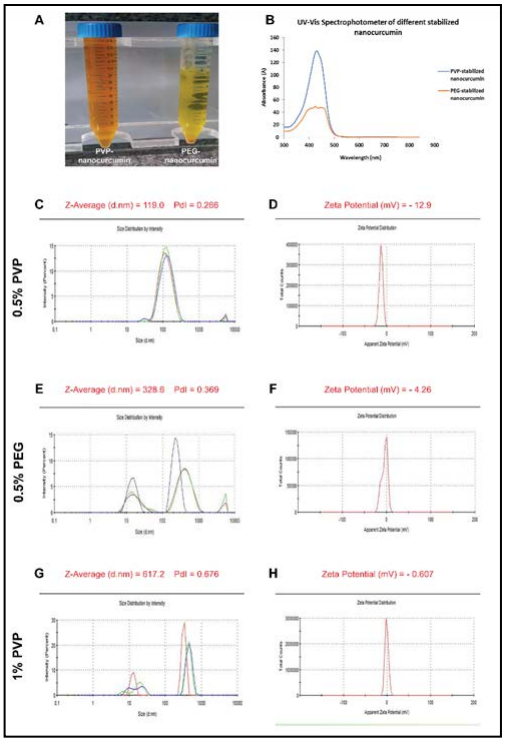

Figure 2. The physical properties, UV-Vis spectrophotometer, and dynamic light scattering results of stabilized nanocurcumin with different concentrations of PVP and PEG. (A-D) Physically, 0.5% PVP gives well-stabilized nanosuspension, in contrast to the precipitate retrieved from PEG coating. Moreover, the PVP-stabilized nanocurcumin shows regular specific absorbance peak with higher-yielding capacity compared to the broad peak of PEG. Furthermore, the nano-sizer and potential results ensures the monomodal uniformly distributed PVP-stabilized nanosuspension, with the smallest particle size, compared to the multimodal dispersion with increased particle size retrieved from 0.5% PEG in (E-F). Increasing the concentration of PVP to 1% does not show improvements either in decreasing or homogenizing the resulted nanoparticles in (G-H).

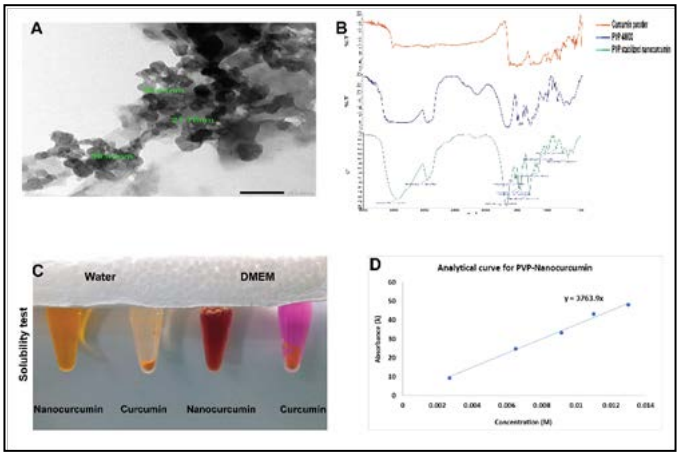

Figure 3. The TEM, FTIR, solubility test, and the analytical curve of 0.5% PVP-stabilized nanocurcumin particles. (A) The TEM reveals spherical monodispersed particles. (B) Comparing between PVP-stabilized nanocurcumin and pure PVP, the FTIR results confirm the success of the coating process. (C) The solubility test reveals the high solubility of nanocurcumin versus curcumin in DIH_2O and DMEM, which results in a highly concentrated nanosuspension of ~36.7 mM calculated from the analytical curve in (D).

Theuse of stabilizers gained our attention to obtain homogeneously distributed nanopopulation of small size. We compared two of the well-known coating agents; PVP and PEG. They provide corona surrounding the nanoparticles preventing their precipitation [25]. However, it was still critical to verify the appropriate amount of stabilizer needed to achieve the optimized nanoparticles' surface coverage ratio.

Upon stabilizing the nanocurcumin with 0.5% PVP, the size decreased significantly, and the nanoparticles became homogeneously distributed. The PVP migrates immediately to the hydrophobic surface of the newly formed curcumin nanoparticles and prevent their growth, resulting in small particle size. On the contrary, coating the nanocurcumin with 0.5% PEG did not reduce the size as PVP at an equivalent ratio. Moreover, it resulted in a poly-dispersed nanopopulation, with broad size distribution. These might be due to the difference in the molecular weight of the used stabilizers, where PEG was 6000, while PVP was 40,000. Increasing the molecular weight of the stabilizer would increase its flexibility and elasticity, which in turn improves the ability of the stabilizer to surround and make a full inclusion of the newly synthesized curcumin nanoparticles inside it [26]. On the other hand, if the polymer length is too short, the nanoparticles would

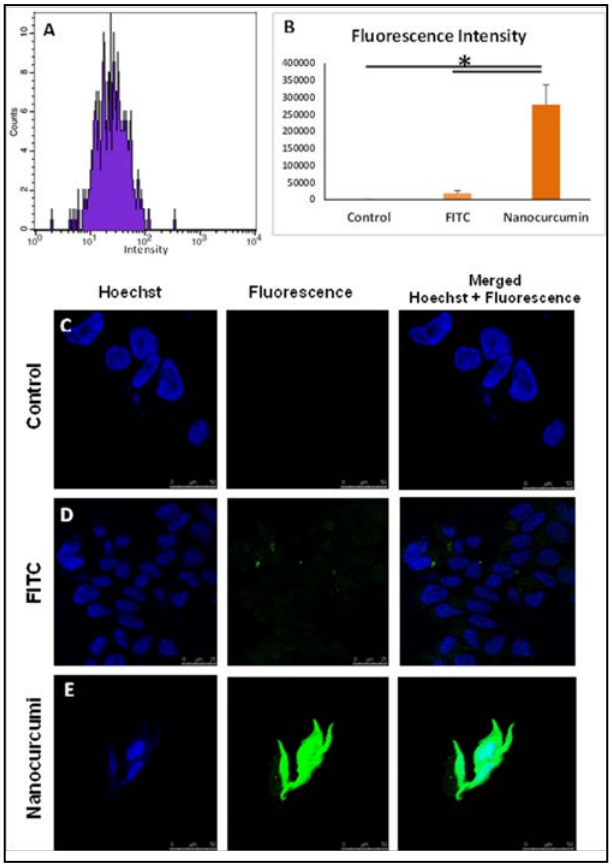

Figure 4. The *in situ* and *in vitro* luminescent properties of nanocurcumin. (A) The flow cytometry reveals the luminescent properties of the curcumin nanoparticles, with a median of 24.14 when exited by blue laser. (B-E) Bar chart and confocal microscopy examination show the intense* auto-fluorescenceof DMEM-soluble nanocurcumin with cytoplasmic and nuclear localization in the gingival fibroblasts, compared to the mild FITC fluorescence of cytoplasmic uptake only. Data in (B) is expressed by mean ± SD and * denotes P<0.000.

not be covered with the polymer layer completely, leading to their agglomeration. Furthermore, the high hydrophobicity of the PEG prevents the adsorption on the nanocurcumin particle surface for steric stabilization [27, 28].

The 0.5% of PVP was sufficient to cover the whole nanoparticle surface and to provide enough steric repulsion between them. Upon increasing PVP concentration to 1%, the mean particle size increased significantly. Similarly, several studies have reported the increase of nanoparticle size, with increasing the amount of the stabilizer [29-31]. The high concentration of the stabilizer would form a thick polymer layer on the particles, contacting them mutually and exacerbating the agglomeration of nanoparticles [32].

In another study, Yadav and his colleagues [16] have used gelatin as a stabilizer in the synthesis of nanocurcumin. However, they concluded that gelatin was effective in arresting the growth of nanocurcumin butdid not prevent their aggregation. Gelatin created a high negative surface charge (-30 mV) around the newly synthesized curcumin nanoparticles, which prevents size growth but allows the aggregation of particles. The PVP in the current study, in contrast, formed an acceptable negative surface charge (-12.9 mV) around curcumin nanoparticles, preventing their growth as well as their aggregation.

The proven water-solubility of synthesized PVP-stabilized nanocurcumin would enhance its bioavailability and cellular uptake, with improving its clinical efficacy. Moreover, the intense auto-fluorescence of the synthesized PVP-stabilized nanocurcumin would help the in-situ tracking of the curcumin nanoconstruct at the cellular level. Mogharbel et al., [13] have utilized the fluorescence properties of curcumin-loaded nanoparticles for tracking cellular therapy in regenerative nanomedicine.Depending on this optical property of the curcumin nanoparticles the herbal nanomedicine will have an added value in the diagnosis besides medication [33, 34].

From our results, we concluded that nanosized curcumin precipitated by stirring acetone-dissolved curcumin and DIH_2O at aslow rate and short time was uniformly dispersed population of high yielding capacity. Furthermore, asmall amount of high molecular weight stabilizer reveals the high stability. Moreover, luminescent property of water/DMEM-soluble nanocurcumin would qualify this herbal nano-candidate to serve as a double theranostic agent, maximizing benefits of one-step therapies.

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