Curcumin and Genistein Coloaded Nanostructured Lipid Carriers: in Vitro Digestion and Antiprostate Cancer Activity

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ABSTRACT: To increase the oral bioavailability of curcumin and genistein, we fabricated nanostructured lipid carriers (NLCs), and the impact of these carriers on bioaccessibility of curcumin and genistein was studied. Entrapment efficiency was more than 75% for curcumin and/or genistein-loaded NLCs. Solubility of curcumin and/or genistein in simulated intestinal medium (SIM) was >75% after encapsulating within NLCs which otherwise was <20%. Both curcumin and genistein have shown good stability (≥85%) in SIM and simulated gastric medium (SGM) up to 6 h. Coloading of curcumin and genistein had no adverse effect on solubility and stability of each molecule. Instead, coloading increased loading efficiency and the cell growth inhibition in prostate cancer cells. Collectively, these results have shown that coloaded lipid based carriers are promising vehicles for oral delivery of poorly bioaccessible molecules like curcumin and genistein.

KEYWORDS: curcumin, genistein, nanostructured lipid carriers, prostate cancer

INTRODUCTION

Curcumin, a hydrophobic polyphenol derived from the rhizome of the herb Curcuma longa, and genistein, one of the isoflavones in soy, are the two most widely studied phytotherapeutics which have shown health promoting and disease preventing activity against several diseases including cancer.2 Among men, prostate cancer accounts for about 29% of total cancer cases in the United States.3 In this connection, both curcumin and genistein have shown superior anticancer activity against prostate cancer both in vitro and in vivo.4,5 In addition, curcumin and genistein in combination have shown an additive therapeutic effect in other disease models.6,7

In spite of a strong antineoplastic activity, curcumin and genistein have not progressed from “bench to bedside” for human use due to their in vitro and in vivo flaws. Curcumin and genistein are sensitive to light, heat, and oxidation, and they suffer from low bioavailability due to less solubility in water (<1 μg/mL), low permeability across the apical surface of intestinal epithelial cells due to low partition coefficient in oil/water, and significant first pass metabolism such as glucuronidation and sulfation.1,8 These flaws pose a number of technological challenges to using them for treatment of cancer. Several studies have shown that the bioavailability of these molecules can be enhanced by increasing the bioaccessibility of these nutraceuticals during the digestion in the small intestine.9,10 Overall, it will be of great therapeutic importance if we develop a delivery system which can protect curcumin and genistein from degradation until it reaches the intestine and in the intestine where it can undergo degradation to form micelles encapsulated with curcumin and genistein thereby increasing bioaccessibility leading to increased bioavailability.

Nanostructured lipid carriers (NLCs) are best suited for the above-mentioned purpose and have an edge over other types of lipid carriers in terms of physical and chemical stability, loading

and entrapment efficiency, and toxicity.11 In the intestine, these lipid nanoparticles undergo lipolysis by various digestive enzymes resulting in the formation of bile-phospholipid micelles.12 Thus curcumin or genistein which has earlier been solubilized in lipid nanoparticles at first consequently solubilized in these micelles and enhances its absorption across intestinal epithelial cells thereby increasing its bioavailability.13

Even though lipid nanoparticles were used for the delivery of siRNA for prostate cancer,14 no effort has been made to develop a lipid based delivery system of curcumin- and genistein-loaded or coloaded lipid nanoparticles for prostate cancer treatment to the best of our knowledge.

Thus, in this study curcumin and/or genistein encapsulated NLCs were fabricated and suitability of the fabricated system for oral delivery was evaluated in terms of their physicochemical characteristics and fate of NLCs in simulated gastrointestinal medium. Furthermore curcumin and genistein loaded NLCs were evaluated for their effect on in vitro cell viability in prostate cancer cell line (PC3).

MATERIALS AND METHODS

Materials. Curcumin (>95% pure) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and genistein with >90% purity was purchased from Macro Care Ltd. (Chungcheongbuk-do, Korea). Glycerol monostearate (GMS) and lecithin from soy bean were purchased from Dae Jung Chemicals (Seoul, Korea). Lipases from porcine pancreas, bile extract porcine and pepsin from porcine gastric mucosa, and pancreatin from porcine pancreas were purchased from Sigma-Aldrich. Dulbecco’s Modified Eagle Medium (DMEM- Phenol red free) was purchased from Welgene (Daegu, Korea). WST-1(4-[

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lecithin were mixed and melted at 72°C following. Curcumin and/or genistein, GMS, oleic acid (OA), and poly(vinyl alcohol) (PVA) at <2 g/100 mL were fabricated by nanoemulsion technique employing high-speed homogenizer and ultrasonic probe as previously reported by our group with slight modifications. The formulations of the NLCs were presented in Table 1. The optimized processes were outlined as follows. Curcumin and/or genistein, GMS, oleic acid (OA), and poly(vinyl alcohol) (PVA) at <2 g/100 mL were mixed and melted at 72–75 °C which forms the lipid phase. The aqueous phase was prepared by adding Tween 80 (T80) to double distilled water which was also heated to 72–75 °C. The aqueous phase was quickly dispersed in the lipid phase and homogenized using an Ultra-Turrax T25 homogenizer (IKA Labortechnik, Staufen, Germany) at 3000 rpm for 5 min. The formed emulsion was further processed (work time 4s, rest time 2s) with a probe sonicator (SON-1VCX130, Sonics and Materials Inc., Newton, CT, USA) for 4 min and then dispersed in cold water containing poly(vinyl alcohol) (PVA) at <2°C which was stirred (600 rpm) for another 15 min. Blank NLCs (BLK NLC) were also fabricated as stated above without adding curcumin and/or genistein to the formulation. The NLCs were lyophilized. All samples were prepared in triplicate. Measurement of Particle Size and Zeta-Potential in Aqueous Solution. Average diameter, polydispersity index, and zeta-potential of the NLCs were measured with a commercial zeta-potential and particle size analyzer (Delsa Nano, Beckman Coulter, Inc., Fullerton, CA, USA) at 25 °C with a scattering angle of 165°. Both size and zeta-potential measurements were performed at least in triplicate (n ≥ 3).

In Vitro Lipid Digestion Assay in Simulated Gastrointestinal Medium. The stability of the fabricated NLCs in simulated gastrointestinal medium were determined by subjecting the NLCs to simulated digestion at pH 2.0 in the presence of pepsin at 37 °C for 2 h, followed by simulated intestinal digestion in the presence of the pancreatin-lipase-bile extract mixture, pH 7.0 at 37 °C for 2 h. Simulated Gastric Digestion. SGM which virtually mimics the conditions in the stomach was constituted as described earlier with slight modifications. A total of 15 mL of blank NLCs (BLK NLC1, BLK NLC2, and BLK NLC3) was mixed with 15 mL of SGM (0.32% w/v pepsin, 2 g of sodium chloride and 7 mL of HCl dissolved in 1 L of water and pH adjusted to 2 using 1 M HCl) and incubated at 37 °C with continuous shaking (μ-strokes/min) in a water bath for 2 h. After 2 h of incubation, samples were used for measuring size distributions and polydispersity index (PDI) with the particle size analyzer.

Simulated Intestinal Digestion. Since digestion of the lipid and micellarization mainly takes place in the small intestine due to the presence of lipase, pancreatic, and bile salts, stability of the fabricated NLCs were studied in SIM as described earlier.17 The NLCs obtained after digestion in SGM were incubated in SIM (lipase 0.4 mg/mL, bile extract 0.7 mg/mL, and pancreatin 0.5 mg/mL) final concentration and 1 mL of calcium chloride solution (750 mM), pH adjusted to 7 ± 0.1 using 0.1 M NaOH at 37 °C and 100 strokes/min. After 2 h of incubation, samples were centrifuged, and the size distributions and PDI of the particles were measured with the particle size analyzer. Entrapment and Loading. Curcumin and genistein entrapment and loading efficiency in the NLCs were studied by completely breaking down the NLCs in methanol and acetonitrile mixture (1:1). The NLCs (1 mL) were added to 9 mL of methanol and acetonitrile mixture and centrifuged at 15 000g for 20 min and the supernatant was used for curcumin and genistein quantification. Agilent-1200 HPLC system controlled by Chem Station software (Hewlett-Packard, Wilmington, DE, USA) equipped with auto sampler and analytical C18 column (Zorbax Eclipse XDB-C18, 4.6 mm ×150 mm, 5 μm packing) was used for quantification. Curcumin and genistein detection was done at ambient temperature using a diode array detector (Agilent g1315D) at wavelengths of 424 nm for curcumin and 261 nm for genistein. Curcumin (20 μL) was eluted isocratically at a flow rate of 0.8 mL/min using a mixture of methanol, acetonitrile, and 5% acetic acid as the mobile phase at the concentration ratio of 35:55:10 (v/v) respectively. Genistein (5 μL) was eluted isocratically at a flow rate of 1 mL/min using methanol/acetonic acid/5% acetic acid/water (25:28:10:37). The entrapment efficiency was determined by using eq 1, and loading efficiency was determined by using eq 2.

\[
\text{entrapment efficiency(%) = } \frac{\text{total nutraceutical added } \times \text{ nutraceutical in the supernatant}}{\text{total nutraceutical added}} \tag{1}
\]

\[
\text{loading efficiency(%) = } \frac{\text{total nutraceutical added } \times \text{ nutraceutical in the supernatant}}{\text{total lipid}} \tag{2}
\]

Curcumin and Genistein Solubilization. Curcumin and genistein solubility in physiological medium was determined by in vitro lipid digestion assay in SIM as explained above (In Vitro Lipid Digestion Assay in Simulated Gastrointestinal Medium section). In addition to curcumin (0.005% w/v) and genistein-encapsulated (0.008% w/v) NLCs, equivalent quantities of curcumin and genistein in their native forms in PBS were also investigated. After 2 h of incubation at 37 °C in the water bath, the micellar fraction which contains the bioaccessible curcumin and genistein were determined as described earlier with modification.18 Briefly, the digesta was centrifuged at 1500 × g for 30 min and the supernatant was collected and recentrifuged at 16 000g for 20 min. A total of 0.1 mL of the supernatant was taken and added to 900 μL of methanol (1:10 (v/v)). Again the solution was centrifuged at 16 000g for 20 min and then curcumin and genistein concentration in the supernatant was quantified from the supernatant by using the HPLC method.

In Vitro Stability. The stability of curcumin and genistein incorporated in NLCs in SIM and SGM without enzymes. The curcumin- or genistein-loaded NLCs were added to respective mediums and incubated in a 37 °C in water bath at 250 strokes/min. At predetermined time points, curcumin or genistein solution was taken and diluted with methanol (1:10 (v/v)) and centrifuged at 16 000g for 20 min, and the supernatant was analyzed for curcumin or genistein content using HPLC.

In Vitro Release. The release studies of curcumin and/or genistein from the NLCs were carried out by using dialysis membrane bags with

Table 1. Compositions of the Blank or Curcumin and/or Genistein Loaded NLCs

<table>
<thead>
<tr>
<th>formulations</th>
<th>GMS (mg)</th>
<th>OA (mg)</th>
<th>lecithin (mg)</th>
<th>T80 (mg)</th>
<th>PVA (mg)</th>
<th>water (mL)</th>
<th>curcumin (mg)</th>
<th>genistein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLK NLC 1</td>
<td>140</td>
<td>60</td>
<td>40</td>
<td>140</td>
<td>20</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BLK NLC 2</td>
<td>140</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BLK NLC 3</td>
<td>140</td>
<td>60</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CUR NLC</td>
<td>140</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>50</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>GEN NLC</td>
<td>140</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>50</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>CUR+GEN NLC</td>
<td>140</td>
<td>60</td>
<td>80</td>
<td>100</td>
<td>20</td>
<td>50</td>
<td>1.5</td>
<td>5</td>
</tr>
</tbody>
</table>

“BLK NLC: blank NLCs; CUR NLC: curcumin-loaded NLCs; GEN NLC: genistein-loaded NLCs; T80: Tween 80; CUR+GEN NLCs: curcumin and genistein coloaded NLCs.”
Table 2. Particle Size, PDI, Zeta Potential, Entrapment Efficiency and Loading Efficiency of the Blank, Curcumin, and/or Genistein Loaded NLCs$^a$

<table>
<thead>
<tr>
<th>formulation</th>
<th>size (nm)</th>
<th>PDI</th>
<th>zeta-potential (mV)</th>
<th>EE (%)</th>
<th>NL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLK NLC 1</td>
<td>149 ± 18</td>
<td>0.30</td>
<td>−43 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLK NLC 2</td>
<td>125 ± 6</td>
<td>0.32</td>
<td>−46 ± 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLK NLC 3</td>
<td>112 ± 5</td>
<td>0.29</td>
<td>−45 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CUR NLC</td>
<td>108 ± 7</td>
<td>0.28</td>
<td>−49 ± 1</td>
<td>78 ± 2</td>
<td>1.2</td>
</tr>
<tr>
<td>GEN NLC</td>
<td>109 ± 8</td>
<td>0.30</td>
<td>−49 ± 2</td>
<td>79 ± 1</td>
<td>2.0</td>
</tr>
<tr>
<td>CUR + GEN NLC</td>
<td>122 ± 6</td>
<td>0.29</td>
<td>−47 ± 2</td>
<td>93 ± 1/82 ± 1$^a$</td>
<td>0.79/2.0$^a$</td>
</tr>
</tbody>
</table>

$^a$BLK NLC: blank NLCs; CUR NLC: curcumin-loaded NLCs; GEN NLC: genistein-loaded NLCs; CUR+GEN NLCs: curcumin and genistein-co-loaded NLCs; EE: entrapment efficiency; NL: nutraceutical loading; PDI: polydispersity index.$^b$Curcumin entrapment or loading in CUR+GEN NLC. $^c$Genistein entrapment or loading in CUR+GEN NLC; mean ± S.D.; n ≥ 3.

6000–8000 Da pore size. The membrane bags were soaked in water overnight ahead of use. The bags were filled with 2 mL of dispersion containing 1200 μg of curcumin in curcumin-loaded NLC, 600 μg of genistein in genistein-loaded NLC, and 700 μg of curcumin and 2100 μg of genistein in curcumin and genistein coloaded NLCs, respectively. The bags were immersed in 18 mL of enzyme free SIM in ethanol (50% v/v) to provide the sink condition and rotated at 50 rpm at 37 °C. A total of 1 mL of sample was withdrawn at predetermined time interval and the same amount of releasing media was replaced soon after withdrawal. The samples were analyzed by HPLC method after suitable dilution.

**Cell Viability Assay.** PC3 cells were obtained from the American Type Cell Culture Collection (ATCC, USA) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS; Welgene, Daegu, Korea). The cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The cytotoxicity of free or NLCs entrapped curcumin and genistein alone or in combination was determined using the cell proliferation reagent WST-1. Briefly, PC3 cells were seeded in triplicate at 5× 10³ cells/well in 96 well plates. After 24 h, the cells were treated with curcumin (20 μM), genistein (45 μM), or curcumin and genistein (20 and 45 μM) either in solution, or in NLCs and the cells was further cultured under the aforesaid conditions for 24 h. At the end of the treatment, the cells were washed twice with PBS to remove the yellow color which was due to the presence of curcumin which may interfere with the final product and 200 μL of fresh medium was added. For the WST-1 assay, 20 μL of tetrazolium salt WST-1 solution was added to each well and incubated for 1 h at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cleavage of WST-1 to yellow formazan was quantified by using a micro plate reader in accordance with the manufacturer’s instructions.

### RESULTS AND DISCUSSION

#### Size Distribution and Zeta Potential

In order to improve the bioavailability via increasing bioaccessibility of curcumin and genistein, curcumin and/or genistein loaded NLCs were successfully fabricated using nanoemulsion technique employing high-speed homogenizer and an ultrasonic probe. All the components used for the fabrication were generally recognized as safe (GRAS) approved (GMS was used as solid lipid and oleic acid was used as liquid lipid. Soy lecithin, T80 and PVA were used as surfactants). Size of nanoparticles plays a crucial role in determining the bioaccessibility of the entrapped molecules by affecting the gastrointestinal tract residence time, dissolution rate and action of digestive enzymes.11,19 Generally particles with <200 nm are preferred for oral delivery. Particle size analysis (Table 2) shows that all fabricated NLCs (blank and nutraceutical loaded) were well within the required range (100 to 150 nm with narrow PDI ~0.3). As the concentration of lecithin was increased, a decrease in size was observed for blank particles. This phenomenon may be due to the formation of additional water/oil interfaces which supports the formation of smaller particles.20 In addition, inclusion of curcumin and/or genistein in NLCs has no effect on particle size and PDI. According to the heuristic rule, surface charge densities (zeta potential) which represents the in vitro stability of the fabricated nanostructures should be more than ±20 mV, that too preferably more than ±40 mV for excellent stability. Surface charge densities of our fabricated NLCs were above −45 mV which clearly indicated the good stability of curcumin and/or genistein loaded NLCs (Table 2). Curcumin and/or genistein loading have no effect on surface charge densities of our fabricated NLCs.

**In Vitro Lipid Digestion Assay in Simulated Gastrointestinal Medium.** Our goal is to first protect the curcumin and genistein from degradation while passing through unfavorable environment in the gastrointestinal tract until it reaches the intestine and then in the intestine to increase the solubilization of curcumin and genistein within colloidal structures like mixed micelles. Mixed micelles consist of bile salts, phospholipids, and lipid degradation products like monoglycerides, fatty acids, etc. Thus, a larger quantity of curcumin and genistein is soluble in these mixed micelles increasing its bioaccessibility.10 To achieve this, interaction of wall materials with the digestive system plays an important role.21 To evaluate the suitability of fabricated NLCs for this purpose, stability of these fabricated NLCs were evaluated in simulated gastrointestinal medium.

Size measurement was used to measure the aggregation or degradation of NLCs because if particle degradation occurs then particle size decreases in the beginning due to loss of surfactant coating on the surface and later size increases due to aggregation of particles owing to lack of surfactants to protect the aggregation.22 In SGM, NLCs were stable and no aggregation or degradation occurred during 2 h of incubation (Figure 1). This stability of NLCs in acidic pH and hydrolysis by pepsin may be due to steric stabilization effect by nonionic surfactants PVA and T80. These nonionic surfactants are indifferent to flocculation and coalescence in low pH due to their molecular structure.23 Similarly, emulsions stabilized by using Tween 20 and Tween 60 with similar characteristics like that of T80 has shown great stability in SGM.24 In addition, acid stable GMS was used as the wall material for the fabrication of NLCs which might have also contributed to the stability of NLCs in SGM.24

After demonstrating the firmness of NLCs in the SGM, stability of NLCs in SIM was studied. Since SIM contained digestive enzymes for which lipids are natural substrates, after 2 h incubation, particle size increased ~4 fold due to particle degradation and aggregation (Figure 1). This aggregation or
degradation phenomenon may be due to hydrolysis of surfactant layer by lipase resulting in particle aggregation and/or by dislodgment of surfactant layer from the surface of NLCs by bile salts or phospholipids in lecithin exposing them for hydrolysis by digestive enzymes. In all of the formulations, lecithin was added as one of the components and bile salt to SIM to maintain the activity of lipase which otherwise is inhibited by monoglycerides and free fatty acids released by lipid digestion. Even though all three blank formulations have shown suitable physicochemical properties in terms of size, zeta potential, and stability in simulated gastrointestinal medium, among the three formulations, BLK NLC3 underwent an abrupt increase in particle size and widening of particle size when stored at 4 °C after 5 days of storage (data not shown). This may be due to the increased mobility of excess lecithin which is left over after thin coating on the NLCs. This excess lecithin leads to the formation of other less stable structures like liposomes, multiple phospholipid layers which collide with each other and fuses with them self- and NLCs resulting in particle instability. Taking all these into account, BLK NLC2 was selected for further experiments. These experiments were anticipated to reveal the best possible combination and concentration of excipients for the production of NLCs.

**Entrapment and Loading.** Since nutraceuticals are required in high concentration to show therapeutic benefits, one of the prerequisites for the successful delivery of nutraceuticals using nanoparticles is high entrapment and loading efficiencies. The fabricated NLCs have shown fairly good entrapment and loading efficiencies (Table 2). Entrapment efficiency of curcumin was 78 ± 2 for curcumin-loaded NLCs and 93 ± 2 for curcumin in curcumin and genistein co-labeled NLCs. In the case of genistein, it was 79 ± 1% for genistein-loaded NLCs and 82 ± 1% for the colabeled NLCs. High entrapment efficiency for both curcumin and genistein is due to their lipophilic nature and also less ordered crystal lattices of GMS which favors accommodation of more guest molecules.

Since curcumin has a high molecular weight, less curcumin was loaded in the lattice defect within the NLCs compared to genistein (Table 2). This clearly indicates that, in addition to lipophilicity (log P = 3.1 for curcumin and log P = 2.9 for genistein), the molecular weight of the molecule also contributes to the loading efficiency. Interestingly, when curcumin and genistein were loaded alone, the loading efficiency with respect to total lipid mass was only 1.2% for curcumin and 2% for genistein. Above this percentage, an increase in size of NLC was observed (data not shown).

However, it was increased to 2.73% (0.7% for curcumin and 2.0% for genistein) when curcumin and genistein was coloaded. This increase in loading efficiency may be due to the difference in the molecular weight of the molecules. When high molecular weight curcumin was added alone it accommodated itself in lattice defects which are big enough to accommodate it, and the remaining lattice defects remained empty. However, when curcumin and genistein were added, as usual curcumin was entrapped within lattice defects which are enough to accommodate it and also genistein was entrapped within the other lattice defects in which curcumin was not occupying. However still more experiments were required to prove the exact mechanism. This data clearly indicates the benefit of coloading of curcumin and genistein.

**Curcumin and Genistein Solubilization.** As mentioned earlier in the Introduction, one of the major hindrances for curcumin and genistein bioavailability is low permeability across the apical surface of intestinal epithelial cells. To overcome this problem we used a lipid based delivery system (NLCs). The intended use of NLCs here is to increase the curcumin and genistein solubilization within the mixed micelles which are formed after lipid degradation in the intestine by digestive enzymes and bile salts. These micelles which are amassed at the surface of the absorptive epithelium are taken up by enterocytes resulting in increased bioavailability. To further increase the entrapment of curcumin and genistein inside the micelles, long chain triglycerides (oleic acid (C = 18) and GMS (C = 21)) were used for the formulation which have shown better efficacy in terms of bioaccessibility compared to medium and short chain triglycerides.

After digestion in SIM, only ~10% of the curcumin and ~25% of genistein were solubilized when added in the form of free powders. When curcumin and genistein were added together in the form of free powders (CUR+GEN sol) ~16% of curcumin and ~20% of genistein were solubilized. The solubility increased up to ~70% for curcumin and up to ~80% for genistein when they were added in NLCs. It is ~90% for curcumin and ~85% for genistein when they were coloaded in NLCs (Figure 2). Coloading of curcumin and genistein did not affected solubilization of each. This increased solubilization is due to the formation of curcumin and genistein entrapped mixed micelles. In addition, GMS which contains ~50%
monoglycerides directly forms the micelles without lipid digestion. In case of free powders, lack of emulsifying agents shuns the successful formation of micelles and also formed micelles were unable to accommodate the large crystals of nutraceutical molecules like curcumin. Recently published data indicated the similar increase in curcumin solubilization using lipid based delivery systems.\(^{18,28}\)

**Curcumin and Genistein Stability Study.** Both in vitro and in vivo stability is one of the major drawbacks that compromises the therapeutic efficiencies of curcumin and genistein. To study the stability of curcumin and genistein, NLCs were incubated in SGM and SIM without enzymes for up to 6 h. Curcumin under these conditions was ~100% stable up to 2 h, but gradually it decreased to ~92% after 6 h. This is in compliance with the earlier results which have shown a similar degradation pattern under analogous condition.\(^{18}\) Genistein degraded faster in these physiological media in comparison with curcumin. It degraded up to 15% within 6 h in SGM and 10% in SIM (Figure 3). A similar degradation pattern of genistein was reported earlier.\(^{29}\)

**In Vitro Release.** The release of genistein and curcumin from NLCs was tested in vitro in enzyme free SIM. To provide the sink condition, 50% ethanol was used. Over a period of 8 h ~61% of genistein and ~55% of curcumin was released from either curcumin or genistein alone loaded NLCs (Figure 4). Overall released amount and rate of the release was always higher for genistein compared to curcumin. This may be due to high hydrophobicity of curcumin (\(\log P = 3.1\)) in comparison to genistein (\(\log P = 2.9\)).\(^{30}\) When coloaded ~96% of genistein and ~67% of curcumin was released which is significantly higher than respective molecules released when loaded alone in NLCs. This increase in drug release may be attributed by the presence of more guest molecules on the shell region compared to curcumin or genistein alone loaded NLCs due to saturation of core region (increased loading efficiency).

**Cell Viability Assay.** Effects on cell viability of PC3 cells after treatment with curcumin and genistein separately or together, either alone or in NLCs, were examined using WST-1 assays. Curcumin at 20 \(\mu M\) had shown ~20% inhibition, and 45 \(\mu M\) genistein suppressed ~12% of cell growth (Figure 5).

When curcumin (20 \(\mu M\)) and genistein (45 \(\mu M\)) were added in the form of NLCs, the cell viability decreased up to 71% for curcumin and 88% for genistein. This increased cell growth inhibition may be due to enhanced intracellular uptake of curcumin and genistein NLCs by the PC3 cells compared to free curcumin or genistein.\(^{31}\) To our surprise ~35% inhibition was seen when PC3 cells were treated with combination of curcumin and genistein NLCs by the PC3 cells compared to free curcumin or genistein.\(^{31}\) To our surprise ~35% inhibition was seen when PC3 cells were treated with combination of curcumin and genistein (20 and 45 \(\mu M\)), and it increased up to 50% when treated with curcumin and genistein-loaded NLCs (Figure 5). As stated above, decreased cell viability may be attributed to the efficient internalization and increased availability of drugs for a longer period. However, the exact reason behind the decrease in cell viability when treated with the curcumin and genistein combination is unknown. Currently we are investigating to elucidate the mechanism of action of this combination in prostate cancer.

**Conclusion.** In the study we have successfully fabricated curcumin and/or genistein loaded NLCs using a high-speed homogenizer and shown the potential of fabricated NLCs for oral delivery of these hydrophobic nutraceutical molecules. The availed results from the in vitro digestion assay clearly indicated the stability of NLCs in SGM and enhanced solubility of...
curcumin and genistein in SIM after encapsulating in NLCs. In vitro cell viability assay revealed enhanced cell growth inhibition by curcumin and genistein after encapsulating in NLCs, which further increased after treating the cells with coloaded NLCs.

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**Notes**
The authors declare no competing financial interest.

**ABBREVIATIONS**
NLCs, nanostructured lipid carriers; SIM, simulated intestinal medium; SGM, simulated gastric medium; PC3, prostate cancer cell line; GMS, glycerol monostearate; DMEM, Dulbecco's modified eagle medium; T80, Tween 80; OA, oleic acid; PVA, poly(vinyl alcohol); BLK NLC, blank NLCs; PDI, polydispersity index; FBS, fetal bovine serum

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