Effects of curcumin-loaded poly(lactic-co-glycolic acid) nanoparticles in MDA-MB231 human breast cancer cells

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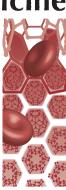
Aim: This study was aimed at evaluating the anticancer potential of curcumin-loaded poly(lactic-co-glycolic acid) (PLGA) based nanoparticles (NPs) in MDA-MB231 human breast cancer cells. **Methods:** Curcumin-loaded PLGA NPs were developed using a modified solvent evaporation technique. Physical characterization was performed on the formulated NPs. Furthermore, *in vitro* experiments were conducted to study the biological activity of the curcumin-loaded NPs. **Results:** Curcumin-loaded PLGA NPs demonstrated high encapsulation efficiency and sustained payload release. Moreover, the NPs exhibited a significant reduction in cell viability, cell migration and cell invasion in the MDA-MB231 cells. **Conclusion:** The study revealed that the formulated curcumin-loaded PLGA NPs possessed significant anti-metastatic properties. The findings showcased the possible potential of curcumin-loaded NPs in the management of debilitating conditions such as cancer. In addition, this study could form the basis for further research and advancements in this area.

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Keywords: anti-metastasis • curcumin • invasion • nanoparticles • PLGA • solvent evaporation

There has been a renewed interest over medicinal plants in the recent decades. Extensive studies have been conducted to substantiate the therapeutic potential of pure natural molecules. Many of these potent natural compounds were targeted to essentially improve the quality of therapeutic regimens against various life-threatening diseases such as cancer [1,2]. The principles of evolution suggest that one species may produce metabolites that may be helpful in the survival of another species [1]. Therefore, these metabolites may, potentially, be used as drugs in the treatment of various diseases. For example, a compound named anagyrine, obtained from *Anagyris foetida*, is an endogenous metabolite found in animals [1,3]. It reveals a close resemblance to a nitrogen moiety present in most neurotransmitters present in animals including humans [3]. This structural resemblance forms the basis to therapeutically employ anagyrine in neurodegenerative diseases [1]. Co-evolution of various species has rendered it difficult and complex, to explain the mode of action of such natural compounds as drugs [1]. However, there







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has been a remarkable progress in understanding the mechanisms of action of such natural drugs. Curcumin is a well-known example of a natural drug which has been used against various diseases such as cancer and inflammation. Curcumin is a naturally occurring compound which is obtained from the plant, *Curcuma longa* [4]. Curcumin has been reported to possess potent therapeutic properties against various pathological conditions and diseases including cancer, neurological disorders, such as Alzheimer's disease, Parkinson's disease and inflammation, mainly due to its anti-inflammatory and antioxidant nature [5,6]. Recent studies have showed that, curcumin may be helpful in preventing cancer by affecting the major glycolytic pathways in cancer cells [4].

The knowledge and utilization of nanoparticles (NPs) and nanocarriers have created a paradigm shift in the way how drugs are delivered to target tissues. Such nanoscale molecules have played a significant role in delivering drug molecules to various tissues and organs. Delivering curcumin using nanocarriers has demonstrated to be a promising therapeutic strategy, as it not only delivers the drug to the target site, but also preserves the therapeutic ability of the loaded drug. In this study, curcumin-loaded NPs were delivered to cancer cells to study their therapeutic ability as an anticancer agent. Various *in vitro* assays such as, cell proliferation assay, cell migration assay and cell invasion assay were performed to assess the effect of curcumin NPs on cancer cells.

Materials & methods

Materials

Poly(lactic-co-glycolic acid) (PLGA) chain terminated polymer (PLA:PGA 50:50, MW 20–30 kDa), curcumin and methanol were obtained from Sigma Aldrich (Dorset, UK), phosphate-buffered saline (PBS) was procured from Oxoid (Hampshire, UK). Dulbecco-modified eagle media (DMEM), fetal bovine serum (FBS), penicillinstreptomycin, optimally reduced serum media, serum-free media and trypsin were procured from Gibco, Life Technologies (Lancashire, UK). Dimethyl sulfoxide (DMSO) was procured from Tokyo Chemical Industry, (Tokyo, Japan). Cultrex[®] 96 well BME Cell Invasion Assay Kit Cell invasion chamber, 5× BME solution, 10× coating buffer, 25× cell wash buffer, 10× cell dissociation solution and calcein AM were purchased from R&D systems, Bio-Techne Ltd. (Oxford, UK). All other chemicals were of analytical grade and obtained from Ulster University store.

Methods

Payload & its characterization using nuclear magnetic resonance

Curcumin is a natural phytopolyphenol substance extracted from the plant, *C. longa*, commonly known as turmeric. The characterization parameters of curcumin-loaded NPs were performed using a proton nuclear magnetic resonance (NMR) method. The curcumin sample was subjected to NMR study, where a 500 MHz spectrometer was utilized to produce its NMR spectra. Briefly, 20 mg curcumin was dissolved in 0.5 ml of deuterated CDCl₃ (chloroform) and the sample was then processed through a NMR instrument. Samples were analyzed against trimethylsilyl chloride as an internal standard [7].

Fabrication of the NPs

Curcumin-loaded nanospheres were formulated by a s/o/w (solid-in-oil-in-water) emulsion technique. Briefly, 50 mg of the polymer (PLGA) was dissolved in 2 ml chloroform. An accurately weighed quantity of curcumin (25 mg) was added to the PLGA/chloroform mixture. The mixture was then sonicated at an energy output of 55 W for 3 min in a Branson Sonifier (model W-350, Branson, CT, USA) to obtain the s/o primary emulsion. This emulsion was then added to a solution of 20 ml PVA (1.5%) and again sonicated at 55 W for 3 min to form the final s/o/w emulsion. The final s/o/w emulsion was left overnight in an uncovered condition with stirring. The mixture was again centrifuged at 13,450 r.p.m. for 15 min to assist the removal of residual solvents. The NPs thus obtained were washed three times with deionized distilled water. The pellet obtained was then finally dispersed in 5 ml of deionized distilled water and eventually freeze-dried (Labconco Freeze Dryer, Mason Technology, MO, USA). The obtained NPs were then stored at room temperature until further use.

Characterization of the formulated NPs – particle size, zeta potential & polydispersity index

The particle size, zeta potential and polydispersity index (PDI) of the formulated NPs were obtained using a photon correlation spectroscopy (Malvern Zetamaster 500 Malvern Instruments, UK) using a 15 mW laser at an incident beam of 676 nm as described by Sharma *et al.*. Briefly, the prepared NPs were filtered through a 0.45 µm filter. The resultant NPs were then dispersed in 1 ml distilled water to determine the size/PDI. For zeta potential

measurements, 1 mM potassium chloride solution was used to disperse the loaded NPs. The mixture was then filtered through a 0.45 μ m filter. The filtered NP sample (40 μ l) along with 400 μ l of distilled water was then filled in disposable folded capillary cells. The viscosity and other parameters, such as temperature and refraction indices, were set according to the solvent which was employed to disperse the NPs.

Quantification of payload

Quantification of curcumin was performed using a UV–Vis spectrophotometer (Varian UV vis spectrophotometer, Mason Technology, NY, USA) via indirect method. Briefly, the supernatant left during the fabrication process of curcumin NPs was used to determine the encapsulation efficiency. The absorbance was measured at a wavelength of 430 nm (λ max). A standard curve was plotted using 1–9 µg/ml of the sample, which was then used to determine the encapsulation efficiency.

Release studies

Release studies were carried out to determine the drug release profile of curcumin-loaded NPs. Briefly, 5 mg of curcumin-loaded NPs were dispersed in 1 ml PBS. The mixture was then incubated under constant end-to-end rotation at room temperature for 96 h. At each time interval, a sample from dispersed NPs was collected and the respective absorbance was measured at 430 nm using a microplate reader. A standard graph of known concentration of curcumin (1–9 μ g/ml) was used to determine the concentration of curcumin released from the sample NPs.

Morphology studies – scanning electron microscopy

Morphology of the curcumin-loaded NPs was determined using a scanning electron microscope (SEM). Briefly, an ultra-thin layer of powdered PLGA–curcumin NPs was applied onto the surface of the metal grid which was later coated with gold and was left aside for 15 min. The sample was then analyzed using appropriate standard set-up parameters in the SEM.

In vitro studies

Cell culture & maintenance

We employed the MDA-MB231 breast cancer cell line in our study. Cells were cultured and maintained according to standard procedures. Briefly, cells were grown in a sterile incubator at 37°C with 5% CO₂. Cells were then harvested and were allowed to become 80–90% confluent in the growth media. Media was then removed and the cells were washed with sterile PBS. Later, the cells were trypsinized and transferred to a new flask containing fresh growth media.

Cell viability assay

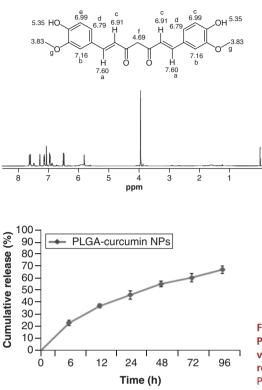
Cell viability was determined by an MTT assay, where 4×10^4 MDA-MB231 cells were seeded in a 24-well plate and later transfected with curcumin-loaded NPs dispersed in the optimal media, using three different concentrations of curcumin-loaded NPs, in other words, 2.5, 5 and 7.5 µg. Cells were transfected for 24, 48 and 72 h. After every 24 h, cells washed with PBS and 500 µl of 10% MTT dye in DMEM were added to each well. Cells were then incubated for 3 h. MTT-containing media was then removed and DMSO was added. The absorbance was subsequently measured at 570 nm using a micro plate reader (Fluostar Omega Multi-Mode Microplate Reader, BMG LABTECH Ltd, Aylesbury, UK).

Migration studies: wound scratch assay

Migration of the cells was determined by a wound scratch assay. Briefly, cells were seeded in a 6-well plate and incubated for 24/48 h until the entire well was confluent. After 24/48 h, a wound was then scratched along the diameter of the well containing cells using a 200 μ l sterile pipette tip. The media was then removed along with the cells that were floating in the media due to the wound scratch. Then, 2 ml of fresh media along with curcumin NPs was added to the cells. Cells were also transfected with blank PLGA-NPs and raw curcumin as controls. Cells were then incubated for 72 h and the image of each well was captured after every 24 h for a total period of 72 h using a compound microscope. The migration distance was then measured using the ImageJ software.

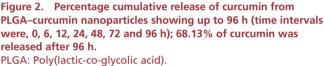
Invasion studies

Cell invasion assay was performed using a Cultrex[®] 96 Well BME Cell Invasion Assay Kit according to manufacturer's instructions. Briefly, the BME membrane was laid in the bottom of the upper chamber and was then



Curcumin NMR estimation

Figure 1. Proton nuclear magnetic resonance spectra of curcumin, where, the lower part of the figure was obtained by a proton NMR, and then was compared with the estimated curcumin nuclear magnetic resonance (sketched using ChemDraw software).



incubated overnight at 37°C. Later, 50×10^4 cells were seeded and transfected with curcumin-loaded NPs along with the addition of 150 µl growth media in the bottom chamber wells of the plate. Cells were then incubated at 37°C for 24 and 48 h. After 24/48 h, media was aspirated from both upper and lower chamber wells. The wells were then washed using a washing buffer. Subsequently, the cell dissociation media mixed with calcein-AM (100 µl) was added to each bottom chamber well to dissociate the invaded cells. The fluorescence of the bottom chamber was eventually measured at 485 nm (excitation) and 520 nm (emission) wavelength using a microplate reader (Fluostar Omega Multi-Mode Microplate Reader).

Results

Payload characterization

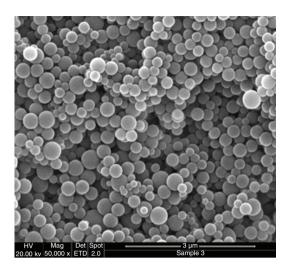
Curcumin was used as a payload, which was successfully encapsulated in PLGA NPs. The structural stability of curcumin was determined using a proton NMR (Figure 1). The obtained NMR spectra of curcumin-loaded NPs revealed a similar pattern as reported by Payton *et al.* [8]. This confirms the structural intactness of curcumin used in our experimental studies [8].

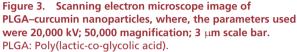
Characterization of the NPs

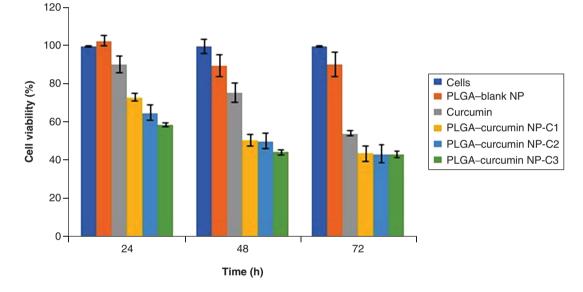
The formulated NPs were characterized using a dynamic light scattering instrument, which was used to determine the average size and surface charge of PLGA–curcumin NPs [9]. The approximate particle size and PDI of the loaded NPs were determined to be 188 nm and 0.319, respectively. The approximate zeta potential (surface charge) was found to be -3.22 mV. The encapsulation efficiency was found to be 79%. Most of the drug was found to be encapsulated within the NPs with a loss of 21% of the drug.

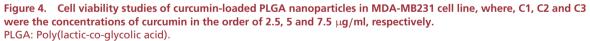
Release studies

Here, the release property of PLGA–curcumin NPs was assessed. Loaded NPs showed a burst release of 23% after the first 6 h, later after 24 h the release of the payload was found to be at 43% (Figure 2). After 96 h, nearly 70% of curcumin was released from the loaded NPs.









Scanning electron microscopy

The morphology of the NPs was assessed using a scanning electron microscope (SEM). The NPs were found to be spherical and smooth as shown in Figure 3. They appeared to be as rigid, round-shaped nano-structures which was an important criterion in terms of fabricating loaded NPs.

Cell viability

The cell viability studies were performed using an MTT assay. Cells were transfected with three different concentrations (2.5, 5 and 7.5 µg) of curcumin-loaded PLGA NPs. Control cells were transfected with blank PLGA-NPs and 7.5 µg raw curcumin. Cells were transfected for 24, 48 and 72 h. After 24 h of treatment, the cell viability of MDA-MB231 cells was decreased to 73% in case of PLGA–curcumin–C1 formulation. The viability decreased proportionally as the concentration of curcumin increased. Cell viabilities were found to be at 65 and 59% for PLGA–curcumin–C2 and PLGA–curcumin–C3 formulation respectively (Figure 4). The reduction in cell viability was due to the burst release of the drug from the NPs which might have killed the cells. Interestingly, raw curcumin was also found to be toxic to the cells where the cell viability was reduced to 90%. After 48 and 72 h, cell viability had further decreased. By the end of 72 h, cell viability has reduced to less than 40% in PLGA–curcumin–C1, PLGA– curcumin–C2 and PLGA–curcumin–C3 formulations. In addition, the IC_{50} value was found to be 4.86 µg/m. In case of raw curcumin, the viability had reduced to 55% after 72 h (Figure 4). Although raw curcumin reduced the viability of the cells effectively but the extent of reduction in cell viability in case of curcumin-loaded NPs was considerably higher than that of the raw curcumin. This strengthens the fact that curcumin-loaded NPs were more effective than the naked drug itself.

Migration studies: wound scratch assay

The cytotoxic action of curcumin was determined by the cell viability study. The anti-metastatic nature of curcumin was studied by performing a wound scratch assay. Three different concentrations of curcumin NPs were used to transfect the cells. The migration of the cells was reduced significantly in case of PLGA–curcumin NPs for all the concentrations studied.

Invasion studies

In our studies, curcumin-loaded NPs were used against MDA-MB231 breast cancer cell line to study the effect of loaded NPs on cell invasion. Cells were treated with 7.5 μ g/ml PLGA–curcumin NPs for 24 and 48 h. In addition, cells were also transfected with blank PLGA NPs and naked curcumin (7.5 μ g/ml). Invasion of the cells was reduced to 80% and 56% after 24 and 48 h respectively in case of PLGA–curcumin NPs. The extent of cell invasion was reduced to nearly half after 48 h.

Discussion

PLGA is preferred over other drug delivery derivatives due to its enhanced drug delivery properties. First, unlike several other derivatives, PLGA is FDA approved which is both biocompatible and biodegradable. This makes PLGA a safer option for drug delivery in either *in vitro* or *in vivo* models as a polymer. Second, due to its user-friendliness and ease of NP fabrication, PLGA makes the whole fabrication process less complex for the researcher. Capped PLGA used in the fabrication of the NPs could be the cause for a lower surface charge. In our study, the charged groups at the ends of PLGA polymer chain was capped, which resulted in a lower zeta potential of PLGA–curcumin NPs. The recorded observations were close to each other, where mPEG-PLGA polymer was used to encapsulate curcumin and fabricate the NPs [9].

Payload release from the NPs could either be monophasic or biphasic depending on the type of polymer used [10]. The sustained release property of the payload from NPs is vital to keep the concentration of the payload constant in the intracellular environment [11]. Khalil *et al.* carried out the release study of curcumin from PLGA NPs where the curcumin release was found to be nearly 45% after 96 h which was comparatively less than the release obtained in our study [12]. This comparatively lesser release of curcumin was probably due to the high molecular weight of PLGA used by Khalil *et al.* Higher molecular weights make the corona of the PLGA more rigid, rendering them less prone to degradation and causing a slow drug diffusion out of the NPs [12].

Poor bioavailability of curcumin has been a long-standing problem [13]. However, with the advent of advanced formulation techniques, this problem could be overcome by embedding the drug into NPs. Our findings revealed that the PLGA NPs enhanced the bioavailability of curcumin which resulted in the destruction of more cancer cells than the raw curcumin [13-15]. In contrast, the blank PLGA NPs caused no cell death. This clearly explains the nontoxic nature of the PLGA NPs. Similar reduction in cell viability has been reported earlier as well [16]. For instance, Chuah et al. prepared curcumin-loaded NPs and then delivered those loaded NPs to HT-29 cell line. The concentrations of curcumin used by Chuah et al. were 10, 20 and 50 µM [14]. In case of curcumin-loaded NPs (10 μ M) the cell viability reduced to 80% after 24 h and on increasing the treatment time from 24 to 48 h and 72 h, cell viability further reduced to 60 and 35%, respectively. In addition, the free curcumin was also found to reduce the viability of the cells significantly [14,17]. This may be essentially due to the cancer cell-destructing effect of the free curcumin. Similar findings were reported by Jithan et al. where, curcumin-loaded albumin NPs were used against MDA-MB231 breast cancer cells to study the cell viability [16]. MDA-MB231 cells were transfected by 100 μ M curcumin for 72 h and the cell viability was reduced to 30%. Whereas, in the case of free curcumin cell viability was found to be 40% [16]. Cell viability observations obtained by Jithan et al. were in corroboration with the findings of our study, performed with PLGA-curcumin NPs [16]. Several studies have previously reported the bioavailability enhancement properties of curcumin-loaded NPs [16]. This in turn has shown to increase the therapeutic efficacy of curcumin. For instance, Thadakapally et al. encapsulated curcumin in PEG NPs and then delivered both the loaded NPs and free curcumin to study the effects of these two forms of curcumin on the

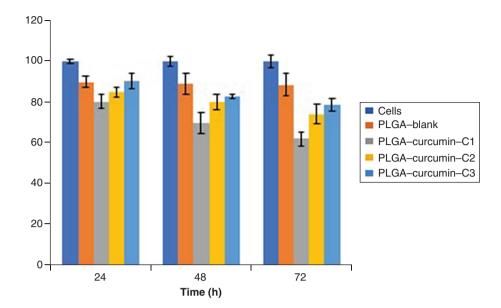


Figure 5. Percentage wound scratch graph of MDA-MB231 breast cancer cells at different time points during migration studies, where, the first bar represents cells with no treatment, second row represents cells treated PLGA-blank nanoparticles, and rest of the bars represent curcumin-loaded nanoparticles in various concentrations (C1, C2 and C3 are 2.5, 5 and 7.5 µg/ml, respectively). PLGA: Poly(lactic-co-glycolic acid).

viability of MDA-MB231 cell line [17]. Their findings revealed that, the encapsulated curcumin inhibited cell viability significantly, compared with the free curcumin [17]. Thus, it may be inferred that, the therapeutic potential of curcumin may be enhanced if delivered along with NPs.

The migration of cancer cells was reduced significantly using curcumin-loaded NPs. On increasing the treatment duration, the rate of migration of MDA-MB231 cells was reduced greatly as compared with the controls (cells alone, PLGA-blank NPs and naked curcumin) as shown in Figure 5. Interestingly, from the images which were captured during the migration studies, it was observed that free curcumin did not contribute significantly towards affecting the migration of the cells (Figures 6 & 7), as stated by Kumari *et al.* [18]. Free curcumin, instead, killed the cells rather than affecting the migration of cells. In contrast, curcumin-loaded NPs slowed down the migration of the cells effectively.

Cell invasion is another important characteristic feature of cancer cells where they invade a target tissue, colonizing them and forming secondary tumors. Essentially, it is important to inhibit invasion of the cancer cells to improve therapeutics against cancer metastasis [19]. The findings from our study support the anti-invasive nature of curcumin. On the other hand, raw curcumin demonstrated invasion inhibition to 60% after 48 h of treatment (Figure 8). Although naked curcumin was found to be effective against invasion, but the extent of invasion inhibition was found to be lesser than loaded NPs. Curcumin-loaded NPs were found to be more effective than naked curcumin. Chen et al. transfected CL-1 lung adenocarcinoma cells with curcumin in various concentrations and demonstrated anti-invasion ability of curcumin against CL-1 cells [20]. After 12 h of treatment, cell invasion was inhibited to 65%, when 5 µmol/l curcumin was used [20]. The percentage inhibition of cell invasion observed in this particular study was higher than the inhibition caused by PLGA-curcumin NPs. This may be due to the high concentration of curcumin treatment used by Chen et al.. A similar reduction in cancer cell invasion was observed where 10 µM, 20 µM and 30 µM concentrations of curcumin were used as a treatment against human endometrial adenocarcinoma cells (HEC-1B cells) [21]. After 24 h of transfection, the percentage invasions of cancer cells were reduced to 80, 50 and 30% when treated with 10, 20 and 30 µM concentrations respectively [21]. Therefore, we infer that curcumin has demonstrated to be an effective agent against cancer cell migration and metastasis. Therefore, in our study, we conclude that curcumin has significantly reduced cell invasion [22-24].

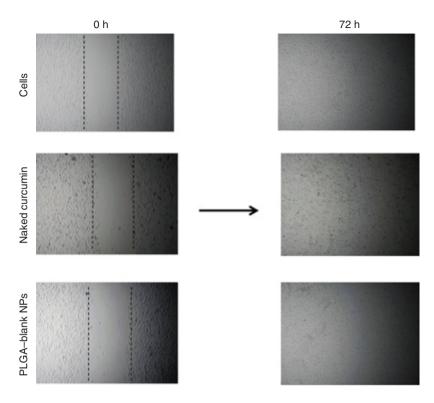


Figure 6. Wound scratch microscopic images of MDA-MB231 breast cancer cells during migration studies, where the first row represents cells with no treatment, the second row represents cells treated with curcumin and the last row represents cells transfected with PLGA-blank nanoparticles. PLGA: Poly(lactic-co-glycolic acid).

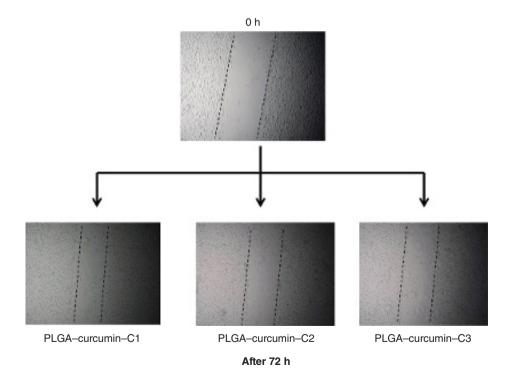


Figure 7. Microscopic image of MDA-MB231 breast cancer cells during migration studies, where the first row represents the cells loaded with PLGA-curcumin nanoparticles at 0 h and the second row represents cells treated with curcumin-loaded nanoparticles in different concentrations (C1, C2 and C3 are 2.5, 5 and 7.5 µg/ml, respectively). PLGA: Poly(lactic-co-glycolic acid).

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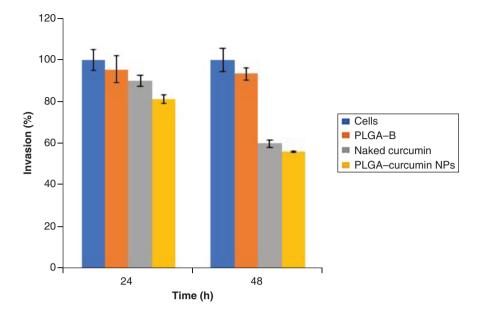


Figure 8. Cell invasion studies of curcumin-loaded nanoparticles on MDA-MB231 breast cancer cell line, where 7.5 µg/ml of curcumin was used to transfect the cells for 24 and 48 h; cells without treatment, PLGA-blank nanoparticles and naked curcumin were used as controls. PLGA: Poly(lactic-co-glycolic acid).

Conclusion

Metastasis is one of the vital characteristics of cancer cells. It is important to control metastasis to improve therapeutics in cancer treatment [25–27]. Curcumin could be a potent natural substance which can be used against metastasis. However, the poor solubility and bioavailability of curcumin have been the major drawbacks in using this drug effectively. NPs have proved to be extremely beneficial in overcoming the challenges posed due to the poor solubility and bioavailability of curcumin. In our study, curcumin was entrapped inside the PLGA NPs. These NPs were then delivered to MDA-MB231 breast cancer cells. Physical characterization of curcumin-loaded NPs showed optimum nano-range size with significant encapsulation efficiency. SEM study revealed smooth, round morphological features of curcumin loaded PLGA NPs. Moreover, the increased encapsulation efficiency of curcumin-loaded PLGA NPs and their improved sustained drug release ability may have increased their therapeutic ability over the previously reported nanosystems. Curcumin was shown to reduce cell viability, cell invasion and inhibit metastasis when the loaded NPs were delivered to MDA-MB231 cells *in vitro*. Based on our observations, we conclude that curcumin is an effective anti-metastatic natural agent.

Future perspective

This study will serve as a platform for newer avenues on the possibility of utilizing curcumin as anti-metastatic agent in MDA-MB231 breast cancer cells. Furthermore, our findings observed in the aforementioned cells will form a basis to extend the knowledge of the in-depth biological mechanisms of curcumin in future studies. In addition, *in vivo* approaches could be used to further assess the therapeutic impact of curcumin.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Summary points

- Metastasis of cancer cells is responsible for the high mortality rate in cancer patients worldwide.
- Natural agents such as curcumin may be used as effective therapeutic agents against cancer cells.
- There is an urgent need to develop effective delivery agents which can protect as well as deliver the desired payload.
- Poly(lactic-co-glycolic acid) based biodegradable nanoparticles (NPs) were fabricated to encapsulate curcumin. The loaded-NPs exhibited smooth surface morphology and nano-dimensional size.
- Poly(lactic-co-glycolic acid) NPs demonstrated a significant and sustained release of curcumin for up to 96 h.
- Introduction of curcumin-loaded NPs resulted in a significant decrease in cell viability. The reduction in the cell viability produced by naked curcumin was significantly lesser than loaded-curcumin NPs.
- Curcumin NPs also significantly inhibited metastasis and cell invasion of cancer cells.
- Naked curcumin showed no effect on metastasis and invasion of cells.
- Curcumin NPs proved to be an effective anti-metastatic and anti-invasive agent against MDA-MB231 human breast cancer cells.

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