**Supporting Information**

**Enhanced allicin cytotoxicity on HEPG-2 cells using glycyrrhetinic acid surface-decorated gelatin nanoparticles**

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S.1. Cytotoxicity evaluation using viability assay

S.1.1. Cells

Mammalian cell lines: HepG-2 cells human hepatocellular cancer cell lines, PC-3 cells human prostate cancer cell line, A-549 cells human lung cancer cell line and MCF-7 cells human breast cancer cell line were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Fetal Bovine serum, DMEM, RPMI-1640, HEPES buffer solution, L-glutamine, gentamycin and 0.25% Trypsin-EDTA were purchased from Lonza, USA.

S.1.2. MTT viability assay

In order to perform the cytotoxicity assays, the tumor cells were dispersed in RPMI-1640 medium at a concentration of 5x10⁴ cell/well (Corning® 96-well tissue culture plates). Afterwards, they were incubated for a period of 24 h. The test formulations were then added into the 96-well plates (three replicates) to reach eight concentrations of each formulation. A number of six vehicle controls with a media of 0.5 % DMSO were added for each 96 well plate as a control. After incubating for 24 h, the exact numbers of viable cells were counted using the popular MTT test. Briefly, the media was removed from the 96-well plates and replaced with 100 µl of fresh culture RPMI 1640 medium without phenol red. Afterwards, 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 ml of PBS) were added to each well including the untreated controls. The 96 well plates were then incubated at 37°C and 5% CO₂ for a period of 4 hours. An 85 µl aliquot of the media was removed from the wells, and 50 µl of DMSO was added to each well and mixed thoroughly with the pipette then incubated at 37°C for 10 min. After, the optical density was measured at 590 nm using a microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and then the percentage of viability was calculated as [(ODt/ODc)]x100 where ODt is the mean optical density of wells treated with the tested sample and ODc is the mean optical density of the untreated cells. The relationship between the surviving cells and the drug concentration was plotted to obtain the survival curve of each tumor cell line after treatment with the specified formulation. The 50% inhibitory concentration (IC₅₀), the concentration required to cause toxic effects in 50% of intact cells, was calculated from the graphical plots of the dose response curve for each concentration using Graph pad Prism® software (San Diego, CA. USA) and applying a non-linear regression fitting model.
S.2. Data statistical analysis

The design of experiments (DoE) and the generation of a response surface methodology (RSM) set of experimental runs and data analysis were performed using Design Expert® v.7.0 (DesignExpert®Software, MN).

An equation representing the model was generated and evaluated regarding R-squared, adjusted R-squared, predicted R-squared and adequate precision. The analysis of variation (ANOVA) study was performed to evaluate the generated model significance. Most of the prepared plain formulae exhibited small PDI of less than 0.4 indicating a narrow particle size distribution and a monodispersity pattern. Hence, only the particle size was the response of choice for statistical modeling. The particle size results were fitted to the generated quadratic model and the efficiency of such model was evaluated by ANOVA test. The p value of the model was < 0.0001 and the lack of fit value was 3.99 which mean that there is only a 14.27% chance that lack of fit, due to noise, can occur referring to a highly significant model.

The generated equation to describe the quadratic model was as follows:

\[ Y = 569.11 - 78.16A - 12.16B + 3.7C + 0.0105AB + 0.0108AC - 0.025BC + 0.67A^2 + 2.48B^2 - 0.00201C^2 \]

Where, \( Y \) is the particle size, \( A \) is the GA%, \( B \) is the cross-linking time and \( C \) is the stirring speed.

Regression coefficient values (R\(^2\)) represent the scattering of the results around the mean proposed by the model. R\(^2\) of value approaching 1 is required. On the other hand, adequate precision is considered the signal to noise ratio. For any obtained model, adequate precision is preferred to be $>4$ in order to be able to navigate the design space.\(^{41}\)

Regarding the data shown in Supporting Information, Table 1, all R\(^2\) values show reasonable agreement with each other demonstrating that the model is highly fitting and powerful to predict the responses accurately. Additionally, the adequate precision was found to be 40.499 indicating an adequate signal to noise ratio.

**Supporting Information, Table 1:** Summary of the results of the regression analysis for the tested statistical design after fitting to the quadratic model.

| Parameter               | Value  |
|-------------------------|--------|
| R- squared              | 0.9965 |
| Adjusted R- squared     | 0.9913 |

S3
It is clear from the 3D surfaces and contour plots in **Supporting Information, Fig. 1, 2 & 3** and ANOVA study that the smallest particle size was obtained at an optimum GA% with an insignificant effect ($p > 0.84$) while when the cross-linking time was increased, the particle size was significantly increased ($p < 0.0001$). This was attributed to interparticular bridging and particles aggregation as clarified in the previous section. Finally, it was found that particle size was significantly affected by the stirring speed ($p = 0.016$). As a conclusion, cross-linking time was proven the most significant factor in the design.

**Supporting Information, Figure (1).** 3D surfaces and contour plots for particle size response to the effect of GA%, cross-linking time at stirring speeds of 700 rpm employed in the second desolvation step of gelatin nanoparticles preparation. The color change from red to blue indicates a decrease in particle size.
Supporting Information, Figure (2). 3D surfaces and contour plots for particle size response to the effect of GA%, cross-linking time at stirring speeds of 1000 rpm employed in the second desolvation step of gelatin nanoparticles preparation. The color change from red to blue indicates a decrease in particle size.
Supporting Information, Figure (3). 3D surfaces and contour plots for particle size response to the effect of GA%, cross-linking time at stirring speeds of 1300 rpm employed in the second desolvation step of gelatin nanoparticles preparation. The color change from red to blue indicates a decrease in particle size.

S.3. Model validation and diagnostics
The validity of the model was assessed by comparing the predicted particle size values produced by the model equation versus the actual particle size values measured after the preparation of GNPs. Moreover, the Box-Cox test for power transformation was also applied as a post-ANOVA test where the natural logarithm of sum of squares of the residuals (ln (Residual SS)) was plotted versus the power of the main response (i.e. particle size) that is named the lambda.

In Supporting Information, Fig. 4 (A) of predicted vs. actual plot, the proximity of points to the 45° line means the predicted values are close to the actual ones reflecting the competence and fitness of the generated model.
For the Box-Cox plot in **Supporting Information, Fig. 4 (B)**, the current lambda (=1) lies within the red confidence intervals (between-2.68 and 4.49) and satisfactorily close to the best power (0.49). Consequently, no further power transformation was recommended confirming the aptitude of such model.

**Supporting Information, Figure (4):** Model validation by: (A) predicted vs. actual plot and (B) Box-Cox plot.
Supporting Information, Figure (5): NMR charts of: (A) glycyrrhetic acid, (B) unconjugated allicin loaded GNPs and (C) conjugated allicin loaded GNPs.