ABSORPTION AND FLUORESCENCE STUDIES OF CURCUMIN BOUND TO LIPOSOME AND LIVING CELLS

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Abstract

The absorption and fluorescence properties of curcumin were used, to study its average binding constants with phosphatidylcholine (PC) liposomes. The liposomal vehicle was examined for the delivery of curcumin to spleen lymphocyte cells, EL4 lymphoma cells and compared with aqueous DMSO vehicles. From these studies it was found that liposomal vehicle is capable of loading more curcumin in to cells than aqueous-DMSO. Lymphoma cells show preferential uptake of curcumin as compared to lymphocytes. The fluorescence of curcumin in EL4 lymphoma cells was found to be significantly higher as compared to the lymphocytes.

Introduction

Curcumin, a natural polyphenol, found in the rhizomes of Curcuma longa (turmeric), exhibits anti-inflammatory, anti-neoplastic, anti-oxidant and chemopreventive activities and has been shown to be pharmacologically safe even at high doses [1-3]. It is a hydrophobic molecule and is practically insoluble in aqueous solutions. Because of this hydrophobic nature, its bioavailability is poor after oral administration and therefore it needs a carrier vehicle to transport it to the desired targets. Curcumin has preferential interaction with lipid membranes [4-5]. Liposome is one of the most commonly used transporting vehicles for drugs, proteins, hormones, diagnostic agents etc [6]. In this paper following absorption and fluorescence changes in curcumin in different systems, quantitative estimations were made on the loading of curcumin from phosphatidylcholine (PC) liposomes to cellular systems.

Materials and Method

Reagents and equipment
Curcumin, cholesterol, acrylamide, potassium iodide, cell culture medium and egg yolk phosphatidylcholine (PC) were procured from local dealers. Whenever
necessary, spectro grade solvents were used. Solutions were freshly prepared in nanopure water from a Millipore Milli-Q system. Mouse spleen lymphocytes were freshly isolated and EL4 cell line (Lymphoma of T cell of mouse origin) was obtained from NCCS, India. Absorption spectra were recorded on a JASCO V-530 spectrophotometer and fluorescence spectra recorded on a Hitachi F-4010 fluorimeter. All the experiments were repeated twice and each experiment was performed in duplicate.

**Preparation of curcumin containing liposome**

Curcumin containing liposome was prepared by dissolving PC, cholesterol and a known amount of curcumin [7] in organic solvent followed by solvent evaporation. The curcumin-loaded liposome was separated from the unbound curcumin through centrifugation. For fluorescence quenching studies, the liposomal curcumin solutions were incubated in quartz cells at 30°C in the presence of iodide (I-) and acrylamide as quenching agents in 10 mM phosphate buffer (pH 7) and the fluorescence intensity at 498 nm was monitored after excitation at 420 nm. The ionic strength of the solution was kept constant at 0.3 M using sodium chloride. This stock solution after appropriate dilution was used for cell uptake studies.

**Estimation of binding constant**

The process of binding of curcumin to the binding agent, BA (BA represents PC liposomes) can be shown by the equations given below.

\[
\text{Curcumin} + \text{BA} \rightleftharpoons \text{Complex} \quad (1)
\]

The equilibrium constant \( K \) for the above equilibrium is given by equation

\[
K = \frac{[\text{Complex}]}{[\text{BA}][\text{Curcumin}]} \quad (2)
\]

For the above equilibrium, assuming 1:1 complex formation, linear plot is made by following absorbance changes at a suitable wavelength, as a function of reciprocal concentration of PC or curcumin according to the equation (3) given below.

\[
\frac{1}{\Delta A} = \frac{1}{K \Delta \varepsilon [\text{BA}]} \left[ \frac{1}{[\text{Curcumin}]} \right] = \frac{1}{\Delta A[\text{BA}]} \quad (3)
\]

Here \( \Delta A \) and \( \Delta \varepsilon \) correspond to the change in the absorbance and the molar extinction coefficient at the wavelength of the study respectively. Using this equation, the values of \( K \) and \( \Delta \varepsilon \) were estimated by following the absorbance changes in curcumin in the region 420 to 450 nm. The binding constants were estimated by following the fluorescence changes too. The changes in fluorescence intensity due to curcumin, were followed as a function of concentration of PC according to equation (4) to estimate the binding constant.

\[
F = \frac{F_0 + F_{\text{complex}} K[\text{BA}]}{1 + K[\text{BA}]} \quad (4)
\]

Here \( F_0 \) and \( F \) are the respective fluorescence intensities from curcumin at a suitable wavelength in the range from 490 to 520 nm in the absence and presence of liposomes and \( F_{\text{complex}} \) is the saturation value.

**Cell culture and uptake studies**

For cell uptake studies, freshly isolated mouse spleen lymphocytes and EL4 cell line (T cell lymphoma of mouse origin) was used. Mouse spleen lymphocytes were isolated as described earlier [8]. For loading of curcumin, the cells were incubated with curcumin for 4 hours. After washing three times, the cells were suspended in Phosphate Buffered Saline (PBS) and subjected to fluorescence spectral studies. The actual concentration of curcumin loaded (uptake) into the cells was determined by extracting cell lysate into methanol. The cellular uptake was expressed in pmoles/million cells.
Results and Discussion

Binding of curcumin with PC liposomes

The absorption spectrum of curcumin is blue shifted from 425 nm to 420 nm in presence of liposomes (Fig.1a and 1b respectively for 1% aqueous-methanol and PC liposomes). The binding constant of curcumin to PC liposomes, was estimated to be $(2.1\pm0.1) \times 10^4$ M$^{-1}$.

Curcumin in aqueous buffer, exhibits weak fluorescence with featureless and broad maximum $\sim$550 nm (Inset a of Fig.1). However with increasing addition of PC liposomes at a fixed amount of curcumin, the fluorescence intensity increased and there is a large blue shift (498 nm) in the fluorescence maximum on curcumin binding to PC liposomes (Inset b of Fig.1). The binding constant for the binding of curcumin with liposomes was also estimated by following fluorescence intensity changes at 498 nm, after excitation at 420 nm to be $(2.9\pm1.1) \times 10^4$ M$^{-1}$. The blue shift in the fluorescence maximum and the increase in intensity with increasing liposome concentration suggest that curcumin in PC liposomes experiences nonpolar environment probably by binding to the hydrophobic regions of PC liposomes.

To know about the distribution of curcumin in different compartments of the PC liposome bilayer, experiments were carried out by following fluorescence quenching studies in the presence of different quenchers at pH 7. Following the quenching of fluorescence from liposomal curcumin by using iodide and acrylamide as quenchers, it is possible to evaluate the location of curcumin inside the membrane. Iodide is a hydrophilic quencher, which can access curcumin in the liposome surface, while acrylamide, being hydrophobic, can access curcumin only when inserted inside the liposome bilayer. The quencher concentrations were varied from 0 to 0.15 M keeping the ionic strength constant. The concentrations of the quenchers and salts were selected in such a way, that they do not induce changes in the bilayer structure of liposomes. The fluorescence data were analyzed according to the Stern-Volmer equation (5).

\[
\frac{F_0}{F} = 1 + K_D [Q]
\]  

Here $F_0$ and $F$ are the intensities of the fluorophore (liposomal curcumin) in the absence and presence of the quencher Q, respectively and $K_D$ is the Stern-Volmer collision constant. Insets of Figs. 2A and 2B show the fluorescence quenching data for iodide and acrylamide respectively and lines show fitting to equation (5). Here, deviation from linearity indicates presence of more than one class of fluorophore, which are not equally accessible to the quencher.
In order to understand the relative population of curcumin in different layers of PC liposome, the fluorescence intensity changes due to curcumin in presence of quenchers were treated with the modified Stern-Volmer equation (6) according to the procedure given in reference [9].

\[
\frac{F_0}{\Delta F} = \left[ \frac{1}{f_a K'_D [Q]} \right] + \left[ \frac{1}{f_0} \right]
\]

(6)

Here \( \Delta F \) is the difference between the fluorescence intensity from the fluorophore in the absence and presence of \( Q \), \( f_a \) is the fraction of fluorophore that is accessible to the quencher and \( K'_D \) is the Stern Volmer constant. Figs. 2(A) and Fig. 2(B) show fitting of the data to equation (6) for iodide and acrylamide respectively. The fitted parameters were found to be \( f_a = 0.22 \pm 0.01 \) and \( K'_D = 10.3 \pm 1.7 \text{ M}^{-1} \) for iodide quenching and \( f_a = 0.40 \pm 0.01 \) and \( K'_D = 31.6 \pm 2.1 \text{ M}^{-1} \) for acrylamide quenching. This confirms that curcumin is non-uniformly distributed into different compartments of the liposomal bilayer and is preferably located inside the hydrophobic interior, which is important for higher drug loading capacity of liposome formulation.

**Cellular uptake studies**

Liposomal formulations were subjected to cell uptake studies for their probable use as delivery vehicles of curcumin to cellular system. To address this, we compared the uptake of curcumin using two different cell systems, spleen lymphocytes and T lymphoma cell line EL4. The cells were incubated with different concentrations of curcumin. Fig. 3 (a-d) shows the representative absorption spectra of methanol extracted cell lysate from spleen cells, after treatment with different concentrations of free and liposomal curcumin. Fig. 3e corresponds to cells treated with DMSO vehicle controls. Other vehicle controls (liposome) showed similar spectra and therefore were not included in the figure. Inset of Fig. 3 shows the amount of curcumin delivered to spleen cells by different vehicles.

Fig. 2 : Plots showing the fluorescence quenching of liposomal formulation containing curcumin in phosphate buffer at pH 7 at constant ionic strength (0.3M) (A) iodide as quencher and (B) acrylamide as quencher. Line shows fitting to equation (6). Insets of Figs. 2 (A) and 2 (B) respectively show the data for the quenching of curcumin fluorescence by iodide and acrylamide fitting to equation (5), dotted lines show linear fits for equation (5).
The figure confirms concentration dependent uptake of curcumin by spleen cells. Similarly, absorption spectra of methanol extracted cell lysate from EL4 cells were also obtained and these confirmed the concentration dependent uptake of curcumin by EL4 cells. After normalization to one nmoles/ml/million cells of curcumin treatment, the average cellular uptake was found to vary in the range of 20-40 pmoles/million cells for these cells. However, it was observed, that the uptake of curcumin is significantly higher with liposomal formulation in either cell type and at the same time cellular uptake of curcumin is significantly higher, in EL4 cells with all the vehicles as compared to splenic lymphocytes. These results indicate that the liposomal formulation is better than aqueous DMSO vehicle for delivering curcumin to cellular system and tumor cells show preferential uptake of curcumin, which is in agreement with the literature reports [1,3]. Fluorescence spectra of curcumin in the above incubated splenic lymphocytes and EL4 lymphoma cells were also recorded.

For these studies, the cell suspensions were excited at 420 nm and fluorescence emission was followed in the wavelength range of 440 to 700 nm. Fig. 4a and 4b show fluorescence spectra of curcumin in splenic lymphocytes treated with free and liposomal curcumin respectively.

Figs. 4c and 4d show corresponding spectra in EL4 cells. The fluorescence from cells treated with vehicle controls showed no detectable fluorescence. Inset of Fig. 4 gives the bar graph showing the comparative fluorescence intensity at fluorescence maximum at 498 nm in these systems.
The results clearly support our previous observations that liposomal system could load more curcumin to both the cell types and EL4 cells show preferential uptake. Interestingly, for the same absorbance at the excitation wavelength, the relative fluorescence intensity was nearly three times more in EL4 cells as compared to lymphocytes suggesting higher fluorescence emission from curcumin in lymphoma cells. The enhanced uptake of curcumin by lymphoma cells could be either due to their high metabolic activity or larger size. The increase in fluorescence intensity in lymphoma cells indicates difference in microenvironment experienced by curcumin molecule inside these cells.

Conclusions

The present study demonstrates the use of absorption and fluorescence methods for quantitative estimation and identification of the loading capacity of curcumin by different vehicles to normal and tumor cells. The absorption method is best suited for the quantitative estimation, while fluorescence method is useful in identifying the site of location. The results confirm that the most efficient means of delivering curcumin to cells is via incorporation in to liposomes and also the lymphatic cancerous cells more readily adsorb curcumin, than normal lymphocytes. Our future experiments are therefore directed to follow this more in detail, to examine whether curcumin can be used for selective imaging of tumor cells.

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References

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