DEVELOPMENT AND CHARACTERIZATION OF MANNOSYLATED QUERCETIN LOADED LIPOSOMES FOR SKIN CARCINOMA

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ABSTRACT: The solar UV radiation is the major trigger factor that causes skin cancer. Nearly 65% of cases of melanoma occurs due to high exposure to the UV radiation. It also accounts for 90% of the nonmelanoma skin cancers also referred to as NMSC also including the cases of basal cell carcinoma (BCC) and the squamous cell carcinoma (SCC). The rate of malignancy in other cases has seemed to get reduced but this is not the situation in case of NMSC, which seemed to have increased and also affect people of younger ages. Cases of NMSC can be accounted for 15,000 deaths in the USA alone. This is surely one of the major health concerns of the nation also. In order to find a solution to this problem a new form of drug delivery system using the specific ligand that has receptor on the surface or the target cells is being proposed. The current discussion deals with the making of the mannosylated quercetin loaded liposomes that can be used for targeting cancerous cells. The methods of transmission electron microscopy (TEM) used for the surface morphology. The MTT assay was done against two different cell lines so that their cancer-preventive or curative properties can be assured. The research, hence establishes the fruitful development of Mannosylated Quercetin loaded liposomes (MA-QuLps) for the targeting of the skin cancer cells.

INTRODUCTION: Skin cancer is an ailment that has attained different types in the last years. The most common type of skin carcinoma is keratinocyte carcinoma. The most common type of malignancy is skin carcinoma worldwide and as deadly as other forms of cancer. Skin cancer is considered as a significant health-related issue which is prominently found in white-skinned population and around 1 million new cases reported of skin cancer in 2018 1, 2. The cutaneous malignancies are represented by nonmelanoma type of skin cancer, and the basal cell and squamous cell carcinoma are collectively known as nonmelanoma skin cancer 3, 4.

The major cause of photocarcinogenesis is the severe exposure of ultraviolet radiation, which can lead to the onset of the prevalent forms of skin carcinoma, basal and squamous cell carcinomas, commonly known as nonmelanoma skin cancers 5, 6, 7. Ultraviolet radiation is considered as a major factor for the induction of permanent alterations in DNA which leads to several modifications on signaling pathways 8, 9.

There are different forms of drug delivery systems have been tried in the past years so that the drug can be applied and delivered to the targeted area that has been affected. However, this has been hindered by various factors like the different mechanisms and tearing at the layers of the skin containing keratin, the interference of the enzymes, and the lipophilicity of the membranes 10, 11. The accuracy and the efficacy of the treatment for skin cancer will depend on the ability of the drug delivery system to reach the affected area that has been cancerous in the skin.
This is how the drug will be delivered to the target cells, overcoming the various barriers and defence mechanism that are present in the human skin. The treatment will be done only in the target cells and not harmed the healthy cells of the skin. The deeper the drug can reach in the skin, the better are the chances of success of the treatment. In the anti-inflammatory treatments, topical delivery system based therapy has been quite successful. This is a treatment type that is further being improvised so that better results can be attained and the side effects can be overcome. The Phospholipids vesicles are already proven vehicles or carriers of the drug delivery system and they can able to protect drug from degradation. They also prevent the drug from interacting with the different biological membranes, improving the systemic and local availability of the drug. Hence, the phospholipids containing lipid vesicle system can lead to better drug incorporation which can improve the effectiveness of the drug as a whole.

In recent times, innovative liposome vesicles are used and the performance of the drugs can be also improved. In the process, better use of extracts from plants is affected. These are the bioactive that possess anticarcinogenic, anti-inflammatory and also antioxidant properties. Mannose receptors require ligand-specific mannose and the mannose receptor is an endocytic receptor that is often present on the macrophage surfaces. It is said to have a profound role in skin cancers caused due to UV radiation. Mannose receptors are overexpressed not only in APCs including DCs but also in macrophages and on dermal fibroblasts and keratinocytes and hence we cannot separate these cells could be without approaching them by targeted compounds for the mannose receptors. Mannose receptors are remarkably expressed in tumor-presenting macrophages that are linked to proliferation, tumor invasion and metastasis in tumor microenvironments. There are several macrophage-targeted delivery systems which is grafted the mannose ligand have been prepared for cancer treatment.

There are various reasons behind the use of these receptors such as

- The presence of the macrophage receptor on the surface of the cancerous cells.
- They are perfect for the delivery of the drug at the right and the best-suited place. Their formulation is also relatively affordable and cheap. Further work and development of them is always a feasible option.

**MATERIALS AND METHODS:**

**Materials:** The sample of Quercetin was purchased from Sigma Aldrich laboratories in Mumbai, India. The Phospholipids used as for source of Phosphatidyl Choline (PC), Cholesterol (Chol) and D (+) Mannose (MA) bought from HiMedia laboratories. The Phosphate buffered saline (PBS) with a pH of 5.5 and 5.6 was used for the drug release studies. The other consumables that were used in the research process had to be of an analytical grade.

**Chemicals for Cell Culture:** The different solvents that were used in the process were of HPLC grade. Millipore super Q water system was used for purification of water. The National Centre for Cell Science, Pune in India provided the two cell lines. The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide for (MTT) assay was obtained from Sigma Aldrich. HaCaT and A-431 cells were cultured in Dulbecco’s Modified Eagle’s Medium Nutrient Mixture F-12 HAM (DMEM F-12 HAM) with 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS), 45 IU/ml penicillin, and 45 IU/ml streptomycin (HaCaT media), at 37°C in 5% CO2.

**Synthesis of QuLps:** The Quercetin loaded liposomes (QuLps) were prepared by slightly modified thin-film hydration method by using a total lipid content of 200 mg at a ratio of PC: Chol of 7:3 that were mixed in 10 ml of chloroform. 20 mg of Quercetin was mixed in 50 ml of ethanol. The solvent was then evaporated at a temperature of 41 °C for an hour. At the end the thin dry film was obtained. The thin film was hydrated using PBS buffer pH- 5.5 at a temperature of 45 °C.

This dispersion was then hydrated for 2 h at the room temperature and then sonicated at the same
environment for thirty minutes. The prepared liposomes were stored in an airtight container and used for further studies 19.

**Attachment of MA with Qu loaded Liposome (QuLps):** The physical adsorption (PA) method was employed to attachment of MA with preformulated liposomes (QuLps) followed here with some minor changes. An acidic buffer was used to disperse the mixture of 20 mg of MA and 2 mg of QuLps. The mixture was stirred for almost 48 h and then collected, washed, filtered and centrifuged. A centrifugal force of 10000 rpm was applied for 20 min at 4 °C. The final product was further lyophilized to get the dried sample 20.

**Physico-Chemical Characterization of MA-QuLps:**

**FT-IR Spectroscopy:** Prepared nanovesicles was evaluated by FTIR spectroscopy. The translucent pellet was prepared by using each sample by grinding and mixing with KBr powder and proceed for scanned over the wavenumber having a range of 400-4000 cm⁻¹ by using FT-IR Spectrophotometer (Shimadzu Bruker Alpha -II FTIR) 21.

**Vesicles Shape, Size and Zeta Potential:** The transmission electron microscope is used to observe the vesicular morphology so that the morphology can be studied and even the vesicular size can be discerned. The dynamic light scattering procedure (DLS) to understand the size distribution of the vesicles. A computerized inspection system is used for the process. The Zeta potential is a measure of colloidal suspension stability 22, 23.

**Encapsulation Efficiency:** For the determination of Entrapment efficiency formulated (Mannosylated Qu loaded Liposomes) MA-QuLps were centrifuged in 15000 rpm for 30 min. The UV-VIS Absorption Spectrometer is used for the analysis (SHIMADZU UV-1800) at 275 nm.

The reference was taken from the curve plotted for various concentrations of Qu in Phosphate-buffered saline PBS having pH of 5.5. On the basis of the standard curve of Qu, the concentration of the drug in the supernatant was deciphered, and the EE% was found using the following formula 24:

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EE\% = \left(\frac{\text{Initial Qu Concentration} - \text{Concentration of unentrapped Qu}}{\text{Initial Qu Concentration}}\right) \times 100
\]

**In-vitro Drug Release Study:** The determination of the Qu release from the mannosylated Lps (MA-QuLps) was done with two different pH conditions that will help the trigger the release of macrophages in the endosomal compartment, and pH 5.6, to match with the physiologic conditions of the skin 25.

**In-vitro Cytotoxicity Assay:** 96-well plates were taken and the cells were seeded into these plates. The density maintained was 1 × 10⁵ cells/well. Samples were prepared in the minimum amount of DMSO and dilution was done using cell culture medium to achieve appropriate concentration according to study protocol to achieve 1000 μg/ml as a working solution. This concentration was maintained in both the cell lines. These cells were kept for treatment for 48 h. 20 μl of MTT stock solution (5 mg/ml) was added at the end and left for an incubation period of 5 h. 200 μl of fresh DMSO was added to each well. The reading was taken at OD 550 nm (absorbance) against an appropriate blank 27.

**RESULT AND DISCUSSION:** Liposomes have been prepared through modified thin-film hydration method following the physical adsorption method for mannose conjugation with preformulated liposomes. The different characterization parameters of MA-QuLps was done. TEM and DLS study were used to assess the morphology and the size distribution of the prepared formulation and entrapment efficiency of the formulation.

**Fig. 1 (A, B)** Qu-loaded Lps revealed a mean diameter in the range of 100-110 nm, and a low polydispersity index (PDI), that suggest that Lps are homogeneous in nature. Statistically, no significant difference was observed on the average diameter of the Qu loaded liposome (117 nm) and that of the MA-conjugated Lps (115 nm). No significant changes take place for MA conjugation in the PDI of the Lps (0.18) when compared to plain Qu-Lps (0.17). Qu-loaded Lps consist anionic surface charge of - 24.9 ± 2.5 mV on their surface as shown in the **Fig. 1C, D.** The physical adsorption (PA) of MA leads to a significant increase in the zeta potential of MA-QuLps (-26.52 ± 1.57 mV). The important factor here is that the thin-film hydration method renders a surfactant-free preparation of Lps.
Surface Morphology: The Fig. 1 (E, F) shows that the TEM image of Quercetin loaded liposomes (QuLps) and Mannosylated Quercetin loaded lipidosome (MA-QuLps). It is seen that they are almost spherical in shape, only the vesicular size is different to some extent depending on the surface texture and composition and their size limited within nanometer range. This was just equal to the order of magnitudes those measured by DLS. The TEM images prove that there are phospholipid bilayers. For the identification of the peaks of Qu, MA, lipids, MA-QuLps, FTIR spectroscopy was used. It confirms that the conjugation between MA-ligand and QuLps. With the help of spectral scan was carried out in (Shimadzu Bruker Alpha -II FTIR). The frequency was limited within 4000 to 400 cm$^{-1}$. The data obtained showed that there was a conjugation that was also confirmed by the featured peaks of lipids. The cholesterol showed some featured peaks at 3514.28 cm$^{-1}$ (Alcohol/Phenol O-H stretching), 2945.10 cm$^{-1}$ (Alkyl C-H stretching) and 1540.38 cm$^{-1}$ (Aromatic C=C bending). The Qu Similarly shows the characteristic $–$OH group stretching at the range of 3700–3000 cm$^{-1}$. Another vital peak important for the distribution of the aromatic ring was found at 1200–900 cm$^{-1}$. In this case, the characteristic peak was detected at 1600 cm$^{-1}$. As shown in Fig. 2G, the conjugation between cholesterol and MA was clear at the peak between 1625 to 1630 cm$^{-1}$.
**In-vitro Drug Release:** The *in-vitro* drug release study was conducted by as per previously reported method. **Fig. 3I** shows the cumulative percentage release of drug. The study was conducted in an environment of physiological pH condition, *i.e.* pH of 5.5 and Acidic pH of 5.6 respectively. In both pH conditions, the drug release profile was measured followed by the same kinetics. There was a cumulative release of almost 70% of preliminary drug loading that was continued for 24 h. During that time, almost 70% of Qu was released. That drug was released from MA-QuLps slowly, and they showed a similar release behavior. That might be the outcome of similar compositions of 5.6 and almost~25% at a pH of 5.5 in 24 h time period. A slower and roughly steady-state (also called lag state) with continued release kinetics was observed during the later stages. The cumulative release of Qu from for MA-conjugated QuLps, the release was found to be 76.43 ± 1.43%, and 72.35 ± 1.58%, for pH 5.6 and pH 5.5 respectively, at the end of 24 h. It was noticed that the drug release was lower when the pH of the release medium was acidic. The results showed that the MA-QuLps were hopefully considered as sustained drug delivery system for future perspective.

**Fig. 2 H,** show the XRD study where the trends for natural flavonoidal substances such as Qu and D-Mannose (MA) physical mixture of Qu, lipids and MA and mannosylated Quercetin loaded liposomes (MA-QuLps) are cleared. The absence of peaks in the XRD pattern for cholesterol makes one thing clear that it is amorphous in nature. On the other hand, natural flavonoid Qu and MA showed some characteristic peak that confirms their crystalline nature. The physical mixtures of MA, Qu, lipids formed different signals where the original peaks were varying, and some new peaks were formed in case of nanoformulation (MA-QuLps). Thus, the results of FTIR revealed that the conjugation between MA and the Lipids. The non-existence of signals related to Qu as a naturally occurring flavonoid confirms that the conjugated forms of liposome possess an amorphous nature when they are inside the lipid matrix.

**Entrapment Efficiency and Loading Capacity:** The Qu could appreciably be entrapped into liposomal formulations, the entrapment efficiency (% EE). The %EE of QuLps and MA-QuLps formulation was found to be 84.08 ± 3.03% and 89.5 ± 0.32%, respectively. High %EE was found because of the lipophilic character of Qu.

**FIG. 3: (I) IN-VITRO DRUG RELEASE OF MA-QuLps AT TWO DIFFERENT pH-5.5, 5.6, (J) EFFECT OF FREE QUERCETIN, QUERCETIN LOADED LIPOSOME QuLps, MANNOSYLATED QUERCETIN LOADED LIPOSOMES MA-QuLps ON CELLULAR VIABILITY (AS % OF CONTROL) OF MTT ASSAY ON HaCaT CELLS (K) EFFECT OF FREE QUERCETIN, QUERCETIN LOADED LIPOSOME QuLps, MANNOSYLATED QUERCETIN LOADED LIPOSOMES MA-QuLps ON CELLULAR VIABILITY (AS % OF CONTROL) OF MTT ASSAY ON A-431 CELLS**
MTT Assay: In the same manner, Fig. 3 (J, K) HaCaT cells and A-431 cells lines were also incubated with diverse concentrations of QuLps, MA-QuLps, and free Qu for 48 h. After doing so, it is investigated that by measuring the absorbance. A reduction in OD550 nm was found for the MTT Assay. The experimental results depicted that in both the cell lines, MA-QuLps exhibited a noteworthy dose-dependent action that was not found so significant in the case of free Qu and QuLps.

CONCLUSION: In the present research work the Mannosylated Quercetin loaded Liposomes (MA-QuLps) were successfully prepared and evaluated as novel targeted drug delivery system for targeting macrophages and the results have been supported the concept that MA-conjugated liposomes have great potential in the drug delivery ability and suitable for improving the targeting approach of lipophilic drug Quercetin. The occurrence of the Mannose on the vesicle shell found to be necessary for targeting macrophages and also an efficient saturation of the liposomes into the skin. However, the amount of the MA moiety present on the vesicle plane considerably affected the flexibility of the vesicles by increasing the packing of the lipid chains and then restraining the structural reorganization of the vesicles. All the data obtained through this study further makes clear the major role exerted by the targeting capability and level of fluidity on skin penetration.

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CONFLICT OF INTEREST: None

REFERENCES:


