Microgels of Hydrophobically Modified-Ethyl Hydroxy Ethyl Cellulose (HM-EHEC) with 5-Flurouracil for Drug Delivery Applications

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Abstract
The polysaccharides (e.g. celluloses and proteins) which form the basic building blocks of life, are gaining increased interest in recent times for researchers to develop newer health care products from renewable bio-polymers which are cheaper and easily available with different desirable properties. Gels are highly swollen three dimensional networks of hydrophilic polymers cross-linked by physical or chemical interactions. Our focus was to design and develop a novel microgel system which would effectively deliver the anti-cancer drug to the targeted site by slow and sustained release for longer times. The water soluble hydrophobically modified ethyl hydroxy ethyl cellulose (HM-EHEC) biopolymer was used in the synthesis of microgels by Michael-type addition reaction between the primary hydroxyl groups of HM-EHEC and Divinyl sulphone (DVS) crosslinker using water-in-oil emulsion technique. The microgels obtained were spherical in shape having flower type morphology with average size of 5 to 8 µm. The anti-cancer drug 5-Flurouracil (5-FU) drug was successfully incorporated and around 56% of the 5-FU was released in 72 hours with a loading efficiency of 95%.

The cell viability (MTT assay) studies confirmed the cycto-toxicity on the MDA-MB 231 breast cancer cell line. There was an increase in the cell death with increase in the concentration of microgel containing drug concentration. The HM-EHEC microgels could be effectively used in the form of a topical cream in the skin and breast cancer for on-site slow and targeted delivery.

Keywords: HM-EHEC, microgels, 5-Flurouracil, drug delivery.

Introduction

According to the World Health Organisation (WHO), cancer is a major cause of deaths in more than 50% of the countries worldwide and a cure for it is one of the biggest challenges of the 21st century. It is a disease which involves the abnormal cells to divide in an uncontrolled manner and destroys the various tissues of the body. The most common type of cancer, with the highest number of cases is the lung cancer which is followed by breast, skin, prostate and colon cancer[2]. Deaths caused due to cancer are estimated to rise rapidly, with an estimated number of more than 20 million by the end of 2025[5]. The diagnosis of cancer must be made in the early stages and must be accurate and require great healthcare facilities and newer advanced technologies. The advancement in nanotechnology for cancer treatment offers a versatile platform for biodegradable and biocompatible systems to deliver conventional chemotherapeutic drugs by increasing their bioavailability and improving the release profiles.

Chemotherapy, radiation therapy, gene therapy, magnetic hyperthermia and targeted therapies are more frequently used for the cancer treatment. However, these treatments have some limitations. Chemotherapy has toxic side effects, by developing resistance to the chemical agents and other forms of treatment are required to be given in combination with chemotherapy. Researchers also report that the various forms of chemo, radiation therapy cause early aging at cellular and genetic level and the cells start to die sooner than normal. Therefore, in this regard, nanogels, microgels/ micro-particles are more frequently being explored as drug carriers for various applications right from diagnosis to therapy[11].

Microgels/nanogels present exciting opportunities with their peculiar physicochemical properties being small in size and possessing high surface to volume ratios. Biopolymer based microgels/nanogels have become an important field in bio-nanotechnology, tissue engineering, medical implants particularly in drug delivery applications[6]. Microgels are three dimensional hydrophilic polymeric networks that are capable of absorbing water and other biological fluids and also help in improving the efficacy of the therapeutic agents and also minimizing the side effects[8]. Polysaccharides based biopolymers like chitosan, alginates, starch and cellulose are commonly used for controlled and targeted drug delivery due to the fact that they are biocompatible, biodegradable.

They have low immunogenicity and are also capable of multitude chemical modifications and therefore biopolymers are a major point of focus in anti-cancer drug delivery applications for diagnostics and chemotherapeutics[10].

The biopolymer reported in this study for the synthesis and characterizations of microgels is based on hydrophobically modified ethyl hydroxy ethyl cellulose (HM-EHEC). It is a non-ionic amphiphilic water soluble polymer with hydrophilic and hydrophobic micro-domains distributed

randomly on the polymer backbone along with low amount of nonyl phenol groups onto the backbone. This polymer is of interest due the fact that it is biocompatible and biodegradable and is suitable for biomedical applications like transdermal/topical drug delivery.

Material and Methods

Hydrophobically modified ethyl hydroxy ethyl cellulose (HM-EHEC, MW 1200KDa, Akzonobel, Sweden under the trade name of Bermocoll (EHM- 500) was used for the preparation of the microgels. Divinyl sulphone (DVS) was used as a cross linker having molecular weight of 118.16 g/mol purchased from Sigma Aldrich, India. The hydrophilic anti-cancer drug, 5-Flurouracil (5-FU) having a molecular weight of 130.07g/mol was purchased from Sigma Aldrich with CAS number 51-21-8 and was used in the controlled release studies in the phosphate buffer saline (PBS) solution.

The obtained HM-EHEC was extracted with acetone for 1 day at room temperature to remove any organic soluble fat. The polymer was then dried and 1.0 wt% aqueous solutions were made. These solutions were centrifuged at 8000 rpm for 40 minutes and the supernatant clear liquid was dialyzed against water using a dialysis bag of 12000 MW cut off (MWCO). The dialysis was carried out for 24 h by frequently changing the external water. The dialyzed solutions were freeze dried to get pure polymer of HM-EHEC.

Toluene, having molecular weight of 92.141 g/mol was the solvent used for microgel synthesis. All the chemicals used were of analytical grade. The cell viability studies were done using the MDA MB 231 human breast cancer cell lines to check the efficacy of 5-FU anti-cancer drug on cancer cells. Distilled water was used throughout the experiment.

The hydrophobically modified ethyl hydroxy ethyl cellulose (HM-EHEC) microgels were synthesized using water in oil (W/O) emulsion polymerization technique. The HM-EHEC polymer (0.5%) was dissolved in 0.1 M NaOH solution. The toluene/water mixtures taken were in the ratio of 3:1 i.e. 30 ml of toluene + 10 ml water. The 10 ml HM-EHEC polymer solution was added slowly to the three neck jar containing toluene-water mixture connected to a water bath. The stirring speed was maintained at 1000 rpm. Within a few minutes of stirring, the toluene water mixture containing polymeric solution turned milky white in colour[1,3]. The initial temperature of the water bath was 25 ^{0}C. When the bath temperature reached 15 ^{0}C, then 30% w/w (i.e with respect to the polymer weight) of divinyl sulphone (DVS) crosslinker was added slowly during the continous stirring.

The Micheal addition reaction of crosslinking takes place between divinyl sulphone (DVS) and the cellulose polysaccharide is shown in fig. 2. The reaction of divinyl sulfone (DVS) with polysaccharides usually leads to cross linked products due to nucleophilic hydroxyl/amino groups attacking the electrophilic double bond of DVS by Michael-type addition[4]. The reaction mixture was kept for 24 hours for complete polymerization and then the reaction mixture was cooled at room temperature. A thick white precipitate was observed. The precipitate was further washed several times with distilled water and centrifuged and the microgels obtained were kept in vacuum oven for overnight drying. The microgel particles were re-suspended in aqueous media and were later freeze dried and the obtained microgel powder was used for the further studies.

The reaction scheme for the synthesis of HM-EHEC microgels is shown in fig. 2 and the experimental cartoon is shown in fig. 3. Microgels are obtained at lower polymer concentration and at higher concentrations, we observe that bulk gels or macrogels and not microgels. We observed at higher polymer concentrations of 1, 1.5 and 2 % (w/v), microgel particles were in micron size (micro-beads) and ranged from 20 to 30 µm. Therefore, the polymer concentration was fixed at 0.5% (w/v) for the synthesis of microgel particles as our aim was to obtain 2 to 10 micron sized particles. The DVS crosslinking density was fixed at 30% w/w. The effects of different crosslinking densities on the size of the micro particles were not studied. We focused on the preparation and synthesis of microgels in the first place.

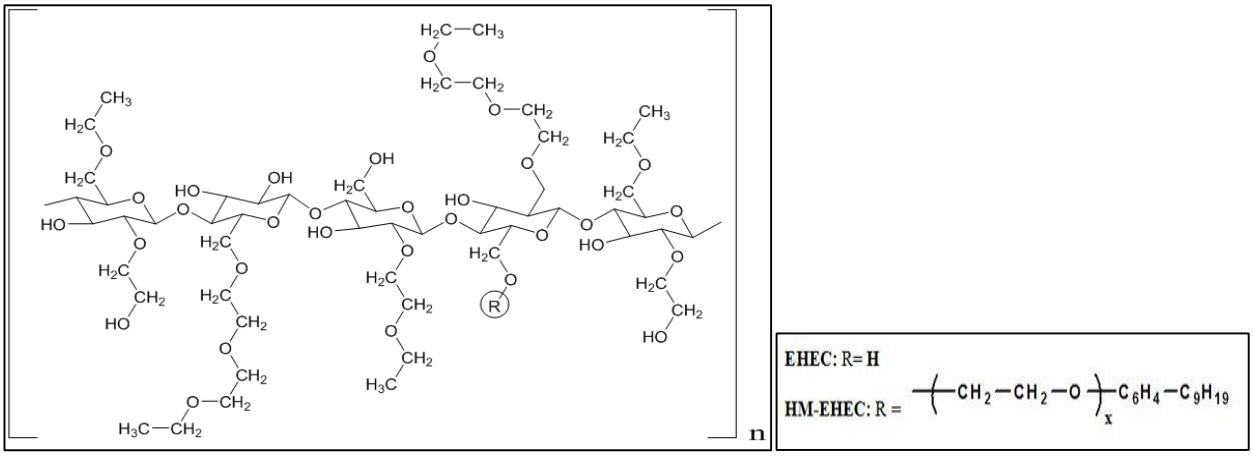


Figure 1: Chemical structure of HM-EHEC.

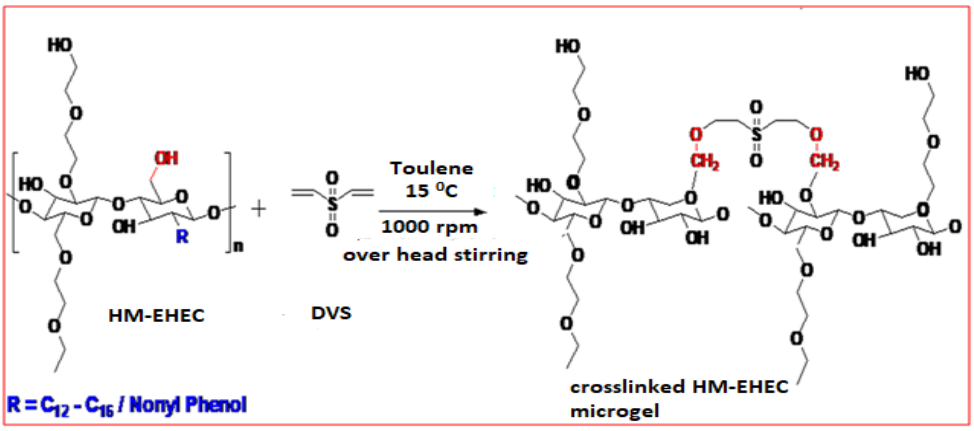


Figure 2: Reaction scheme for formation of microgels.

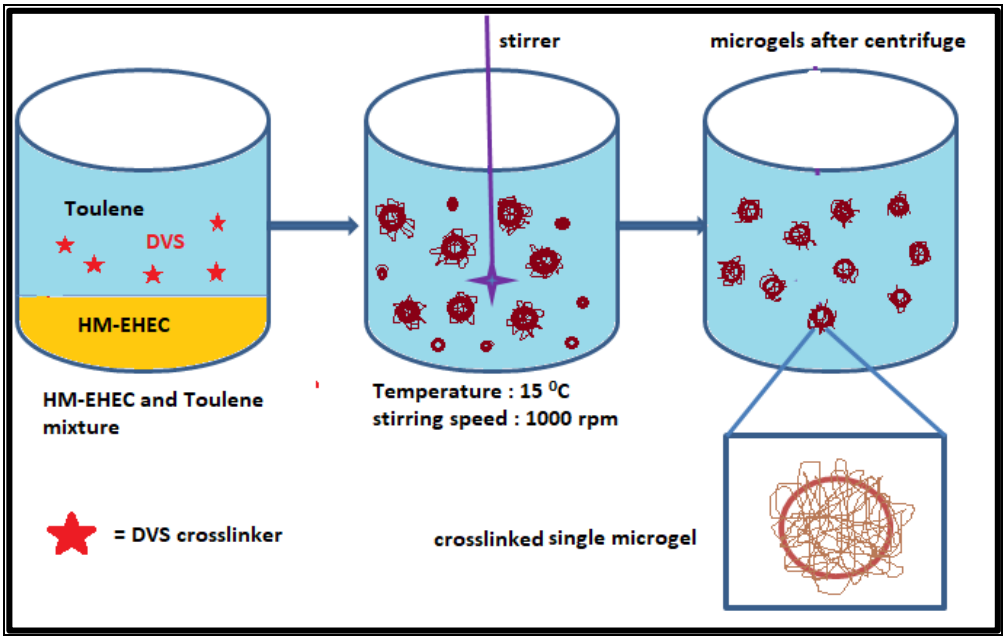


Figure 3: Water in Oil (W/O) emulsion technique for microgel synthesis.

The increase in the crosslinking density would produce rather hard and rigid microgels which would not be so effective for our application and moreover the drug loading and the swelling capacity would be varied. However, lower concentrations of 5, 10 and 20% (w/w) of DVS crosslinker were tried but from the SEM images, it was clear that there was no uniform formation of the microgels, rather coagulation and aggregation of the particles were observed.

Scanning Electron Microscopy (SEM): The morphology of the nanofibers was studied using Scanning Electron Microscopy quanta 200 3D dual beam ESEM (FEI, Finland). The electron source was tungsten (W) filament with thermionic emission at 15 kV in high vacuum. Before SEM imaging, the microgels were sputter coated with a thin layer of gold.

Fourier Transform Infra-red spectrometer (FT-IR): The IR spectra of the nanofiber mats were recorded on Perkin Elmer Instruments, Spectrum One, FT-IR spectrometer. The recording was done in a diffused reflectance mode in the wavelength range of 400 to 4000 cm^{-1}. The samples were mulled with KBr powder and the background scan was done with pure KBr (Potassium Bromide) disc for recording the spectra.

***In vitro* 5-FU Drug Release:** The 5-Flurouracil drug release from the microgels was studied in phosphate buffer saline (PBS). A 15 ml phosphate buffer solution (PBS) was taken in test tube which was covered with an aluminium foil and kept in a shaker bath (Julabo SW 23) with gentle shaking at 37^0C. Known quantities of microgel particles (5mg) containing 5-FU were placed into the test tube. At predetermined time intervals, 1 ml of PBS was taken and replenished with an equal volume of fresh PBS into the test tube. The amount of 5-FU released was determined by UV-Vis spectrometer (UV-160 PC Shimadzu) at a maximum absorbance wavelength λ_{max} of 266 nm[13]. The percentage drug release was calculated in PBS at 7.4 pH. The release experiments of each sample were performed in triplicate and the average values were reported. The encapsulation efficiency was calculated using equation:

Encapsulation efficiency (%) =

$$\frac{\text{mass of maximum drug released}}{\text{mass of total drug released}} * 100$$

***In vitro* cyto-toxicity study:** The cyto-toxicity study of the HM-EHEC microgels was performed on the MD-MBA 231 human breast cancer cell line, using MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The cancer cells were obtained from National Centre for Cell Science (NCCS), Pune, India. The cells (4×10^4) were seeded in 24-well, flat bottom culture plate and incubated for 24 h. After incubation, the MTT solution (10 mg/ml of stock solution of which 20µl of MTT solution was added in 100µl of DMEM media) was added to each well followed by further incubation at 37^0C for 4 h. The formazan crystals formed were dissolved by addition of acidified isopropanol. After 15 min, the amount of coloured formazan derivative formed was determined by measuring the optical density (OD) using a microplate reader (Spectra Max, MS; Molecular Devices, LCC) at 570 nm.

Microscopic images of the well plates were obtained at 0 and 24h as control and treated samples. All the experiments were done in triplicate. The percentage cell viability was calculated by the equation:

$$\% \text{ Cell viability} = \frac{(OD)\text{sample} - (OD)\text{blank}}{(OD)\text{control} - (OD)\text{blank}} * 100$$

Results and Discussion

Scanning Electron Microscopy (SEM): The SEM images of the HM-EHEC microgels are shown in fig. 4. The SEM micrographs show that the microgels are spherical in shape having a coarse surface. The microgels are seen well dispersed and the average diameters of the microgels are in the range of 5 to 8 µm. The images show a spherical flower like morphology using the HM-EHEC polymer. This morphology was checked and repeated several times and the same flower like morphology was obtained each time. The emulsion technique used for microgel synthesis using the natural water soluble biopolymer namely HM-EHEC connects the hydroxyl groups in the cellulose by forming a 3D hydrophilic network via a covalent bond by crosslinking with di vinyl sulphone (DVS) which might have helped in obtaining flower-like morphology.

Fourier Transform Infra-red Analysis (FT-IR): The structural changes in the microgels were confirmed by the FT-IR analysis after the incorporation of 5 FU anti-cancer drug. The prominent peaks of 5-FU were quite diminished due to the fact that encapsulated drug existed in the amorphous state into the microgel matrix. Fig. 5 shows the spectra, where a broad band between the 3136 cm^{-1}, is attributed to the N-H stretching vibrations of 5-FU.

The peak at 1413 cm^{-1} belongs to C-F stretching band in the spectrum of 5-FU. The peak at 1247 cm^{-1} is due to C-N stretching vibrations of 5-FU. The peak at 1351 cm^{-1} refers to vibration of pyrimidine compound confirming 5-fluorouracil. The peak at 1320 cm-^{1} refers to the stretching vibration of S=O which is merged with 1351 cm^{-1} with the 5-FU drug[7,12].

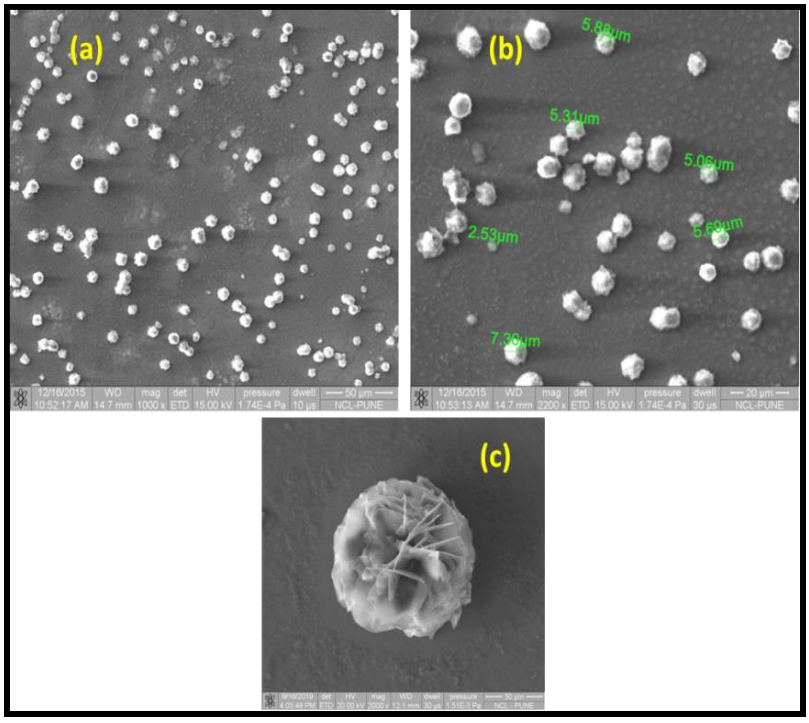


Figure 4: SEM images of HM-EHEC microgels.
(a) Scale bar: 50µm; magnification 1000 X (b) Scale bar: 20µm; magnification 2200 X
(c) Scale bar: 30µm; magnification 2000 X.

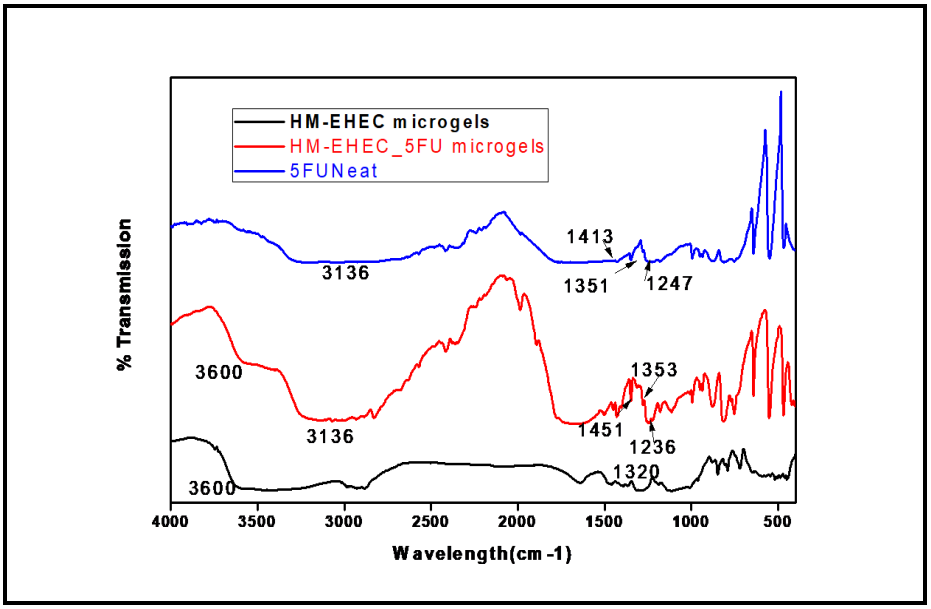


Figure 5: FT-IR spectrum of HM-EHEC microgels along with 5-FU.

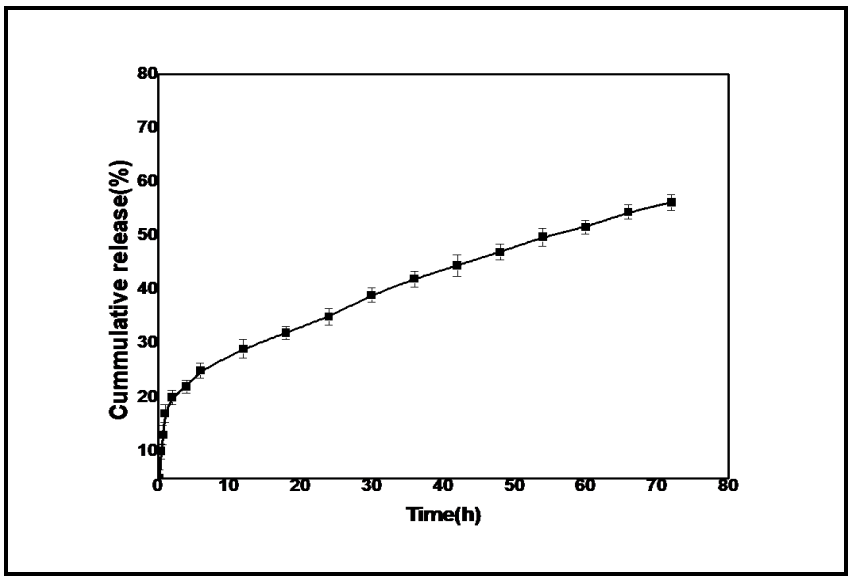


Figure 6: Drug release of 5-FU from the HM-EHEC microgels.

Drug release of 5-Flurouracil (5-FU): Microgels have been used as drug delivery vehicles for low molecular-weight to bio-macromolecules. The binding of these drugs is greatly influenced by the local crosslink density and the functional group distribution within the microgel[15]. The *in vitro* release profile of the drug 5-FU is shown below in fig. 6. The 5-FU drug release studies were carried out at 37 ^{0}C. The microgel samples (5 mg) were incubated in a PBS solution in an incubator with a shaking speed of 200±10 rpm. The encapsulation efficiency was found to be 95% (passive loading). The initial burst release accounted for 20% ±2 during the first 2 hour of the release study. The total amount of the drug released in the next 72 hours was found to be around 56%.

In the process of loading the drug into the microgel system, there could have been some losses and therefore the real encapsulation efficiency could have been higher than the actual percentage of the drug loaded. Nevertheless, a slow and controlled release of 5-FU was observed over the time. The uneven distribution of the drug in microgels often results in initial burst effect for few minutes and then exhibits slow and sustained release for several days to weeks[9].

Cell viability studies (MTT Assay): The cell viability of human breast cancer cell line was studied for the toxicity effect by the 5-FU drug. The MDA MB 231 cells ($4x10^4$) were seeded into 96-well plate and incubated further at 37ºC. These cells were either treated or untreated with microgels containing the 5-FU for 24 hours. After 24 hours the cells were incubated with MTT (0.5mg/ml) for 4 hours to form the formazan crystals. The formazan crystals formed were dissolved in isopropanol and the absorbance was measured at 570 nm in ELISA reader (Thermo Scientific).

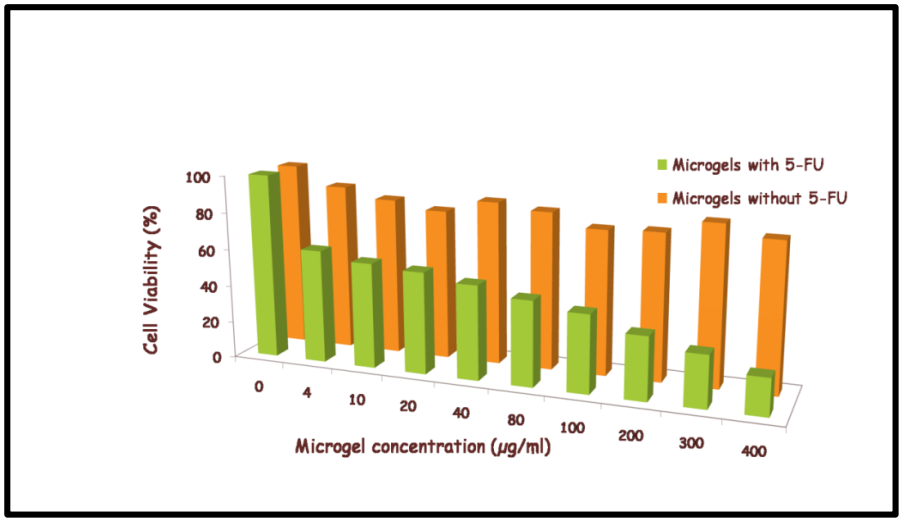
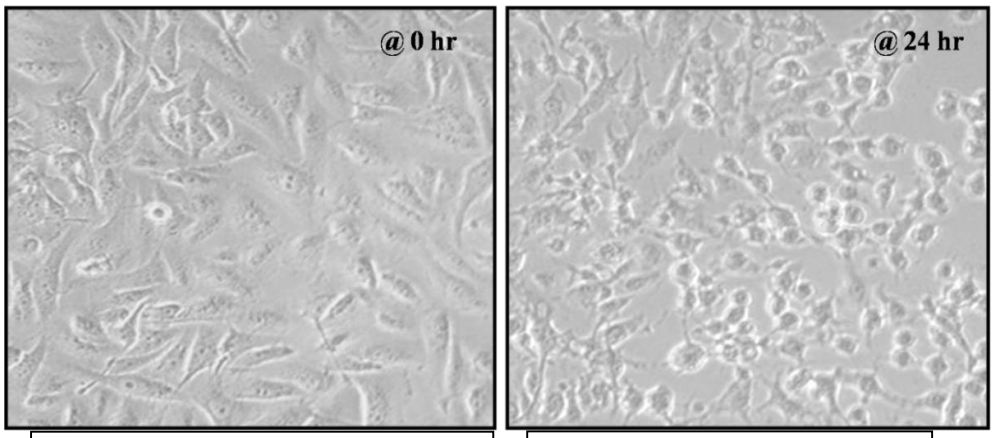


Figure 7: MTT Assay for HM-EHEC microgels with and without 5-FU.



a) Untreated with 5-FU **b) Treated with 5-FU**

Figure 8: Confocal Microscopy images of MDA-MB 231 cancer cells.

All the experiments were performed in triplicate, analyzed statistically and represented graphically in the form of bar chart as shown in the fig. 7. The cancer cell line photographs before and after treatment were taken using Image Pro plus 6.0 software under phase contrast microscope (Nikon) with magnification 20 X as shown in fig. 8. The images reveal that before the treatment, the cells maintained their morphology and after the treatment with 10µg/ml microgels containing 5-FU, the cells lost their elongated spindle shape morphology and were seen round in shape after 24 hours which indicates the cell death[14]. The cell proliferation was inhibited and cell death occurred due to the anti-cancer drug. More cell deaths were observed with increase in time and concentration of the microgel loaded 5-FU.

Conclusion

In this chapter study, we aimed at synthesizing polymeric based microgels for topical drug delivery of an anti-cancer drug for a site specific application on the skin. We could successfully synthesize the HM-EHEC microgels by using water in oil emulsion technique. The average size of the microgles was found to be in the range of 5 to 8 µm. The microgels were spherical in shape and uniformly distributed with a flower-like morphology. The 5-Flurouracil drug was incorporated into the microgel matrix which was confirmed by FT-IR analysis. The anti-cancer drug loading efficiency was 95% for the 5% drug incorporation.

The study revealed the initial burst release for 2 hours where 20% of the total drug loaded was released and then there was a slow and controlled release of 56% drug observed upto the next 72 hours. The MTT assay was done using the MDA-MB 231 breast cancer cell line to check for the cell viability and cyto-toxicity of 5-FU on the cell lines. The cell death increased with the microgel concentration and it was observed that 50% cell death occurred when 100µg/ml of microgel concentration contained around 12µg/ml of 5-FU. The microscopic images of the treated and untreated microgels also exhibited the cell death of the cancer cells.

Acknowledgement

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