CHAPTER-4

Materials and Methods

4.1 Materials

4.1.1 Chemicals

SNo.	Chemicals	Source
1	Capecitabine	Cipla Ltd. Bangalore, India
2	Locust bean gum	Sigma-Aldrich, USA
3	Sodium alginate	Central Drug House (P), Ltd. New Delhi, India
4	Calcium chloride	Sigma-Aldrich, USA
5	Aluminum chloride	Sigma-Aldrich, USA
6	Potassium dihydrogen phosphate	SD Fine Chemicals, Mumbai, India
7	Sodium hydroxide pellets	Merck Specialties Private Limited, Mumbai, India
8	Heparin sodium injection	Biological E. Limited, India
9	Hydrochloric acid	Hi Media, Mumbai, India
10	Methanol (HPLC grade)	SD Fine Chemicals, Mumbai, India
11	Sodium carbonate	SD Fine Chemicals, Mumbai, India
12	Disodium hydrogen phosphate	SD Fine Chemicals, Mumbai, India
13	Membrane filters (0.45 µm)	Hi Media, Mumbai, India
14	Dimethyl sulfoxide (DMSO)	Thermo Fisher scientific Private Limited, India
15	Dulbecco's Modified Eagle Medium	Hi Media, Mumbai, India
16	Fetal bovine serum	Hi Media, Mumbai, India
17	Trichloroacetic acid (TCA)	Hi Media, Mumbai, India
18	Potassium chloride	Ranbaxy laboratories Ltd. Punjab , India
19	Sodium chloride	Hi Media, Mumbai, India
20	Acetonitrile (ACN)	Merck Ltd., Mumbai, India
21	Ammonium acetate	Hi Media, Mumbai, India

22	Sodium bicarbonate ($NaHCO_{3)}$	Hi Media, Mumbai, India
23	L-glutamine	Hi Media, Mumbai, India
24	Ethanol	Merck Ltd., Mumbai, India
25	Formaldehyde	Merck Ltd., Mumbai, India
26	Acetone	Merck Ltd., Mumbai, India
27	Potassium dihydrogen orthophosphate	Qualigens Chemicals, Mumbai, India
28	Dialysis membrane (MWCO 12-14 kDa)	Hi Media, Mumbai, India
29	Petri plates	Hi Media, Mumbai, India
30	Aluminum foil	Hindalco,Sonbhadra,India
31	Laboratory film	Parafilm, Chicago

4.1.2Equipments

S No.	Instruments	Source
1	Digital magnetic stirrer	IKA®, USA
2	Digital electronic balance	Axis, Poland
3	Digital pH meter	IKON Instruments, New Delhi, India
4	Cooling centrifuge	REMI C20, Mumbai, India
5	Digital microscope	Dewinter optical. Inc., India
6	Electric Oven	Cintex, Mumbai, India
7	Disposable syringes	Hindustan Syringes & Medical Devices Ltd., Faridabad, India
8	Microplate absorbance reader	Bio-Rad iMark™, USA
9	CO_2 Incubator	Sanyo CO_2 Incubator, Japan
10	Sonicator (Bath type)	WUC, Fisher Scientific, India
11	UV-Visible Spectrophotometer	Shimadzu, Japan
12	FTIR 8400S	Shimadzu, Japan
13	Vortex mixer	REMI Instruments, Mumbai, India
14	HPLC	Shimadzu, Kyoto, Japan
15	Micropipettes	Eppendorf, Germany
16	Dissolution apparatus (Type II)	Electrolab, India

17	Scanning electron microscope	Carl Zeiss, USA
18	Powder X-Ray Diffractometer	Bruker D8, Germany
19	Attenuated Transmittance Reflectance (ATR)	Shimadzu, Japan
20	Triple distilled water assembly	Milipore, USA
21	Gamma (γ camera)	Varicam, Panasonic, Japan

4.1.3 Animals and Cell lines

S No.	Animals and Cell lines	Source
1	Albino Wistar rat	Central Animal House, Banaras Hindu University, India
2	Swiss Albino Mice	Central Animal House, Banaras Hindu University, India
3	HT-29 (colon cancer cell line)	ACTREC, Mumbai, India
4	BALB/c mice	ACTREC, Mumbai, India

4.1.4 Software

S. NO.	Software	Source
1	Minitab-17	Conventry CV3 2TE,UK
2	Design Expert® version 11	Stat-Ease, USA
3	Origin Pro 8	MA, USA
4	Dewinter Biowizard	Dewinter, India
5	Kinetica 5.1 ™	Lancaster,CA, USA
6	GraphPad prism 7	California, CA, USA

4.2 Pre-formulation studies

Prior to the formulation development, pre-formulation studies are performed to detect any change in the characteristics of the drug and its suitability during formulation preparation.

Preformulation studies include

- Physical appearance
- Melting point
- FTIR
- Analytical method development of CAP by UV-visible spectroscopy
- Analytical method development of CAP by HPLC in plasma

4.2.1 Physical appearance

The drug sample was observed for its organoleptic property.

4.2.2 Melting point

The melting point of CAP was determined by using scientific melting point apparatus (Gallen camp).

4.2.3 Fourier transform Infrared spectroscopy (FTIR)

The spectral analysis of pure CAP was done by FT-IR spectrophotometer (Shimadzu 8400, Japan). For the study pinch of the sample drug was taken and crushed with dry potassium bromide in mortar pestle followed by its compression into a pellet by pressed pellet technique. The prepared pellet was then placed in the sample holder and analyzed in the spectral region between 4000-500 cm^{-1}.

4.2.4 Identification of CAP by UV spectrophotometric method

UV visible spectrophotometer was employed for the identification of CAP. The quantitative estimation of CAP was performed in simulated intestinal fluid or phosphate buffer (SIF; pH 6.8) and simulated gastric fluid 0.1 N hydrochloric acid (SGF pH 1.2).

4.2.4.1 Preparation of phosphate buffer (pH 6.8)

Composition: Potassium dihydrogen phosphate- 28.8 g; Disodium hydrogen phosphate 11.45 g; Distilled water upto 1000 mL.

Phosphate buffer (pH 6.8) was prepared as per Indian Pharmacopoeia (IP), 2014. Briefly, 28.8 g of potassium dihydrogen phosphate and 11.45 g of disodium hydrogen phosphate was dissolved in distilled water to produce 1000 mL.

4.2.4.2 Preparation of 0.1 N hydrochloric acid (pH 1.2)

Composition: Hydrochloric acid (12N) - 8.33 mL, Distilled water upto 1000 mL**.** Preparation of 0.1 N hydrochloric acid of pH 1.2 involved addition of 8.33 mL of concentrated hydrochloric acid in distilled water to produce 1000 mL.

4.2.4.3 Preparation of stock solution of Capecitabine and its solubility determination in two *in vitro* release media

a) Preparation of stock solution of Capecitabine

100 mg of CAP was weighed and dissolved in 100 ml of each buffer media separately (pH 1.2 and 6.8). The stock solution was further diluted with the same solvent to give another stock solution of concentration 100 µg/ml. Finally, from the above stock solution aliquots of 1, 2, 3, 4, 5, and 6 mL were withdrawn and the volume was made up to 10 mL to obtain the concentration range of 10-60 µg/mL. Drug solution in the respective mediawas scanned and the UV spectrum was recorded from 200 to 400 nm.

b) Determination of solubility

Solubility of CAP was determined in triplicate, in both the *in vitro* release media of pH 1.2 and pH 6.8 by shake flask method. Excess amount of drug CAP was added separately to 5 mL of each solvent contained in a glass vial. The samples were stirred on magnetic stirrer for 24 h. The formed suspensions were filtered to remove excess of undissolved drug. Further, the solutions were diluted with their respective media (pH 1.2 and pH 6.8) and assayed for drug content using UV spectrophotometer at 239 nm.

4.2.5HPLC bio-analytical method development and validation

4.2.5.1 Preparation of standard solution

The parent stock solution (1 mg/mL) of CAP was prepared by dissolving 10 mg of drug into 10 mL of methanol from which series of 10-500 µg/mL of working standard was obtained by further diluting the stock solution with methanol and water mixed in a ratio of 50:50 v/v [Dhananjeyan et al., 2007].

4.2.5.2 Preparation of sample in plasma

The plasma was collected as a supernatant after centrifuging the blood obtained from Albino Wistar rats. The sample preparation was done by adding 100 µL of methanol to 100 µL of plasma that leads to the precipitation of the sample which was further centrifuged at 5000 rpm for 10 min at 4°C. The obtained clear supernatant was transferred and reconstituted in 100 µL of the mobile phase. Finally, an aliquot of 20 µL was injected into HPLC column after filtering the sample with 0.45 µm milipore filter [Xu and Grem, 2003].

4.2.5.3 Preparation of mobile phase

The mobile phase for the study was constituted of methanol and phosphate buffer (pH 6.8) in a ratio of 70:30. Preparation of phosphate buffer (pH 6.8) was done according to subsection 4.2.4.1. The mixture utilized for mobile phase was filtered through 0.45 µm membrane prior to the use.

4.2.5.4 Chromatographic condition

The bioanalytical method validation of CAP was carried out on HPLC (Shimadzu, corporation, Kyoto, Japan) comprising UV-Visible detector (SPD-20 A), pump (LC-20AD), rheodyneannual injector (SIL-20A). Estimation of the drug was done at 239 nm by injecting the samples in a C-18 reverse phase column (250 mm x 4.6 mm, 5µ Enable). The analysis was performed under an isocratic condition with mobile phase methanol and phosphate buffer (pH 6.8) (70:30) at a flow rate of 1 mL/min at wavelength 232 nm.

4.2.5.5 Recovery, linearity, precision and accuracy

The % recovery of the drug was determined by spiking equal amount of drug into the blank plasma sample and was calculated by comparing the peak areas of CAP from sample extracts to the peak areas of absolute responses of non-extracted standard i.e. CAP in methanol. The standard curve of CAP in plasma was constructed with six different concentrations in the range of 10-500 µg/mL. Each sample was injected in triplicate and the curve was plotted

between drug concentration and a peak area of the CAP. Precision was calculated by injecting the six replicates of plasma samples of the concentration range 100 and 500 μg/mL between and within the day and was expressed as a percentage of relative standard deviation (RSD). The acceptable limit of precision reported is ≤ 15% for all concentration [Hassanlou et al., 2016]. Accuracy was calculated from the same samples used during inter and intraday study. It was expressed as % bias with an acceptable limit of ± 15% at each concentration [Zufıa et al., 2004].

4.2.5.6 Limit of detection (LOD) and quantitation (LOQ)

LOD is defined as the lowest concentration of the analyte in the sample that can be detected but not necessarily quantitated as an exact value. It resultsin a peak area of 3 times to the baseline ratio. LOQ is defined as the lowest concentration of the analyte in the sample that can be quantitatively estimated by exact precision and accuracy. For estimation of LOQ, the peak area with a signal to noise ratio must be greater than 10, where precision should be less than 15% (RSD) and accuracy within ± 15% (bias).

PART I

4.3 Development of Locust bean gum (LBG) and Sodium alginate (NaAlg) based Calcium ion (Ca^{2+}) cross-linked Interpenetrating Polymeric Network (IPN) loaded with Capecitabine (CAP); (F-1)

4.3.1 Risk assessment study

As per the current regulatory science philosophy, Quality by design (QbD) is a necessary key element applied during several pharmaceutical developments[Pallagi et al., 2015]. Application of QbD has brought systematic, scientific risk-based and proactive method for product development [Woodcock, 2004]. According to International Conference on

Harmonization (ICH) Quality Guidelines, QbD can be defined as “a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control,based on sound science and quality risk management” [Rathore and Winkle, 2009]. The steps in QbD assisted product development involves:

- Determination of quality target product profile (QTPP), that includes patient related product performance and quality characteristics of the drug product [Gandhi and Roy, 2016]. QTPP elements include a route of administration, delivery system, dosage form etc. [Guideline, 2009].
- Determination of critical quality attributes (CQAs) deals with the physical, chemical and microbiological property that must lie in the appropriate limit, range or distribution to achieve the desired quality product. It includes drug substances, excipients, intermediates (in-process material) and drug products [Guideline, 2009].
- Determination of critical process parameter (CPPs) and critical material attributes (CMAs). They both affect CQAs thus, must be observed and controlled to ensure the quality of the product [Guideline, 2009].
- Application of pre-optimization study, involving screening of possible CQAs and CPPs of major concern through screening study (Fractional factorial design) that may affect CPPs.
- Application of optimization study involving the most critical factors having the most influential effect on CPPs.

Another most important term Risk assessment (RA) is equivalent to QbD and aims to keep away the risks from occurring. It systematically observes and controls CMAs and CPPs. In the present study risk ranking and filtering method has been used to assess and manage the risk. Risk Ranking and Filtering (RRF) are one of the most common facilitation methods used for Risk Management. This method is also known as “Relative Risk Ranking,” “Risk

Indexing," and "Risk Matrix and Filtering." Its intent is to provide a sharper focus to the critical risks within a system – typically, from a large and complex set of risk scenarios. RRF works by breaking down overall risk into risk components and evaluating those components and their individual contributions to overall risk. The entire risks (CMAs and CPPs) termed as independent variables that can affect CQAs or response were categorized and ranked according to the severity (S) and probability (P). Table 4.1 illustrates the rating of S and P. Finally, CMAs and CPPs scoring 10 or >10 were considered as high-risk factors and exhibited in Table 4.2.

Table 4.1 Risk ranking according to their severity and probability

Risk	**Rank**	**Classification**	**Criteria**
Severity	4	High	A small change in the parameter can influence the quality of the product.
	3	Moderate	Varying parameters may cause loss or influence the product quality.
	2	Low	The quality of the product remains operable.
	1	No effect	
Probability	4	High	High chances of failure
	3	Moderate	Fewer chances of failure
	2	Low	Failure unlikely
	1	No effect	

Table 4.2 Risk Ranking and Filtering

Independent variables(CMAs and CPPs)	**S x P**	**RPN**	**Criteria**
➢ API	2 x 3	6	Moderate
➢ Polymer	3 x 2	6	Moderate
➢ Cross- linker	3 x 3	9	Moderate
➢ Solvent	1 x 3	3	Low
➢ Polymer amount	4 x 4	16	High
➢ Cross-linker amount	3 x 4	12	High
➢ Preparation	3 x 2	6	Moderate
➢ Characterization	4 x 2	8	Moderate
➢ In vitro study	4 x 2	8	Moderate
➢ Stirring speed	4 x 3	12	High
➢ Dropping distance	4 x 4	16	High
➢ Relative humidity	3 x 2	6	Moderate

➢ Magnetic bead size	2 x 2	4	Low
➢ Beaker size	2 x 1	2	Low
➢ Temperature	2 x 4	8	Moderate
➢ Calibration error	4 x 2	8	Moderate
➢ Curing time	4 x 2	8	Moderate
➢ Needle gauze	3 x 4	12	High
➢ UV/FTIR/SEM/XRD	4 x 2	8	Moderate
➢ Magnetic stirrer	2 x 4	8	Moderate
➢ Probe sonicator	3 x 2	6	Low
➢ Electron microscope	3 x 2	6	Low

4.3.2 Screening of the influential factors: Fractional Factorial Design

Fractional factorial design (FFD), an important statistical design technique is well known for its efficient investigation of factors on the response of interest. Though several methods are there that reduces the number of trials in the experiment however in contrast to them the size reduction in an experiment done by FFD provides an optimal result as well as the consequences while reducing the trials in the experiment thus, mostly preferred for screening purpose [Gunst and Mason, 2009]. In the present study, five factors screened out by RA were incorporated into FFD. The coded factors (A, B, C, D, and E) were set at two levels (+1) and (-1) respectively Table 4.3. Finally, the selection of the critical factors was done on the basis of the obtained Pareto chart where only the critical factors crossing the reference line were taken into account. The entire screening study was done with the help of Minitab-17® software.

Table 4.3 Screening of risk factors by fractional factorial design

Runs	**A**	**B**	**C**	**D**	**E**	**Y_1 (µm)**	**Y_2 (%)**
1	+	−	−	−	−	510.67	80.34
2	+	−	+	−	+	515.31	77.19
3	+	+	−	+	−	496.18	85.51
4	+	+	+	+	+	500.23	81.60

5	−	−	−	+	+	354.29	66.26
6	−	+	−	−	+	341.50	73.59
7	−	+	+	−	+	338.77	70.13
8	−	−	+	+	+	360.43	63.60

A= polymer ratio, B= amount of cross-linker, C= stirring speed, D= needle gauze, E= dropping distanced

4.3.3 Optimization and statistical analysis

In the current study, three factors, three levels with six center points Box Behnken Design (BBD) was carried out using Minitab-17® software. Application of BBD can markedly limit the number of experimental runs with an increase in the number of parameters [Qiu et al., 2014]. BBD is the most effective and efficient design technique that helps in the determination of first and second order coefficient of mathematical [Bezerra et al., 2008]. Selection of the independent variables influencing the development of IPN microbeads was the basic requirement of the experimental design. The essential and the fundamental material *viz.* polymer blend, cross-linker and process variable namely stirring speed were chosen as influential variable screened out finally by FFD. IPN is a concoction of polymeric blend whose network is intercalated with each other by a cross-linker thus; selection of the amount of polymer blend and cross-linked directly influences the development of IPN. A process variable, stirring speed decides the size of the IPN beads which also affects directly or indirectly polymeric network. Hence for the present study, the amount of polymer blend (A), amount of cross-linker (B), and stirring speed (C) were selected as independent variables. With respect to independent variable particle size (Y_1) and % drug entrapment (Y_2) were selected as responses. Both these responses affected the quality of the IPN microbeads. Based on statistical data for instance lack of fit test, regression– square value (R^2), p-value etc. a suitable model was selected and further with respect to the best model polynomial equation

was generated predicting the effect of independent variables on the dependent variables. Finally based on response constraints an optimized batch generated was used for entire *in vitro* and *in vivo* characterizations [Singh et al., 2016].

4.3.4 Preparation of Ca^{2+} cross-linked IPN encapsulating CAP using natural polymers LBG and NaAlg

CAP loaded IPN microbeads of natural polymers LBG and NaAlg were prepared by ionotropic gelation method [Jana et al., 2016]. Briefly, CAP (50 mg), LBG and NaAlg (1:1 outof1000 mg total weight of polymers) were dispersed in distilled water and stirred on a magnetic stirrer at 500 rpm for complete and proper mixing. The prepared mixture then poured drop-wise through the 23-gauze needle into another beaker containing calcium chloride (4% w/v) with continuous stirring at 100 rpm for 30 min. The developed IPN microbeads were washed and rinsed constantly with water and dried at room temperature. A diagrammatic stepwise preparation of IPN carrying CAP is shown in Figure 4.1.

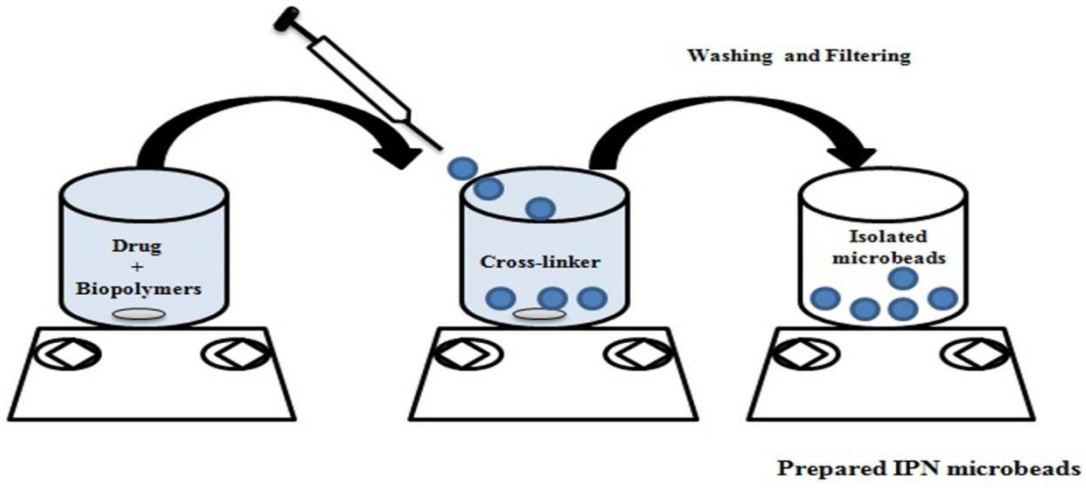

Figure 4.1 Preparation of divalent ion (Ca^{2+}) cross-linked IPN microbeads

4.3.5 Characterization

4.3.5.1 Determination of particle size

4.3.5.1.1 Optical electron microscopy

The particle size of microbeads was determined by the method reported earlier [Yadav et al., 2017]. Before estimation of particle size, the eyepiecemicrometre was calibrated with stage micrometre and then, approximately 100 dried IPN microbeads were randomly selected and placed on stage micrometre. The average size of the microbeads was measured using Edmond's Equation

$$Averageparticlesize = \frac{\sum numberofmicrobeads \times meansizerange}{\sum meansizerange}$$

4.3.5.1.2 Scanning electron microscopy (SEM)

Surface morphology of the microbeads was observed on SEM (Supra 40, Zeiss, Japan). Preparation of sample was done by mounting and adhering microbeads on aluminium stubs with double side adhesive tapes followed by sputter coating with gold palladium under argon environment using high vacuum evaporator.

4.3.5.2 Energy Dispersive X-ray (EDX)

Estimation of the composition and level of elements present in the prepared formulation was done on EDX instrument (Ametek, NJ). The study was done by scanning and focusing on the dense region of the formulation by X-ray radiation at a fixed angle.

4.3.5.3 Fourier Transform Infra-Red spectroscopy (FTIR)

To investigate the possible interaction among drug, polymer, physical mixture and prepared formulation, FTIR (Shimadzu 8400, Japan) study was performed. For sample preparation briefly, a pinch of all sample materials was collected and mixed separately with potassium bromide. Finally, the samples were turned to pellets by pressed pellet technique. The formed pellets were scanned between 4000-500 cm^{-1}.

4.3.5.4 X-ray Diffraction (XRD)

XRD spectrum of pure drug, polymers LBG, NaAlg, physical mixture and formulation was recorded on Rigaku portable X-ray diffractometer (Rigaku, Japan). The study determines the crystalline state as well as change in the physical state in the formulation during the fabrication process [Pan et al., 2012]. Samples were exposed to Cu-K radiation at 35 kV voltage with a current of 30 mA. All the samples were scanned between 5°-60° at 2θ at a step size of 0.01° at 2° min scanning speed.

4.3.5.5 Differential scanning calorimetry (DSC)

DSC study was done to analyze the change in the phase transition of the sample by uptake of heat energy [Gill et al., 2010]. The study was conducted on DSC instrument (DSC Q20, V24.11 Build 124, USA). Each sample i.e. CAP, LBG, NaAlg, physical mixture and formulation weighing approximately 5 mg were kept on aluminium pans in an airtight or hermetically sealed condition. The samples were scanned at a heating rate of 10° C within the range of 0-250° C under nitrogen environment flowing at 60 mL/ min.

4.3.5.6 Drug entrapment (%)

Drug entrapment of CAP loaded IPN microbeads was determined by the method reported previously [Banerjee et al., 2013]. Approximately weighed 100 mg of dried IPN microbeads were weighed and transferred to 100 mL phosphate buffer and sonicated for 10 min. Afterwards, the solution was filtered through milipore membrane filter (0.45 µ membrane filter) for removal of polymeric debris. The filtrate was estimated on UV spectrophotometer (Shimadzu, 1800) at 239 nm. The drug entrapment was calculated using formula

$$Drug\ entrapment\ (\%) = \frac{actual\ content\ of\ drug\ in\ microbeads}{theoretical\ content\ of\ drug} \times 100$$

4.3.5.7 Swelling study

The swelling study was performed to interpret the movement of liquid into the microbeads [Agnihotri and Aminabhavi, 2006]. Briefly, 50 mg of IPN microbeads were initially placed in 10 mL of 0.1 N HCl for 2 h followed by phosphate buffer (pH 6.8) for 3 h. The data of swelling for each media was recorded at different time interval by removing the particles from the respective buffers and wiping the extra liquid by blotting with tissue paper. Swelling index was calculated by using the following equation

$$\text{Swelling index (\%)} = \frac{\text{weight of swollen microbeads} - \text{weight of initially dried microbeads}}{\text{weight of initially dried microbeads}} \times 100$$

4.3.5.8*In vitro* drug release study

In vitro drug release study was performed in two buffer media (pH 1.2 and pH 6.8 respectively) by dialysis bag diffusion method [Jha et al., 2011]. The dialysis membrane (HiMedia Laboratories, Mumbai, India; molecular weight cut off 12-14 kDa) was filled with 100 mg microbeads (equivalent to 500 mg of pure drug) tied at the end of the paddle of dissolution apparatus (Electrolab, India) and immersed in 900 mL of buffers. The apparatus was maintained at 37 ± 0.5 °C and the paddle was rotated at 50 rpm. After stipulated time intervals sample withdrawn at each time were analysed on UV spectrophotometer (Shimadzu, 1800) at 239 nm for estimation of the drug release.

4.3.5.9*In vitro* cell cytotoxicity study

SulforhodamineB (SRB) assay was performed to evaluatethe cytotoxicity activity of CAP loaded optimized formulations in human colon cancer cell line HT-29 procured from ACTREC, Mumbai, India by following the method reported earlier by Vichai et al. [Vichai

and Kirtikara, 2006]. The cell lines were incubated in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and 2Mm L-glutamine. andinoculated into 96 well plates incubated with 5% carbon dioxide, 95% air at 37°C (100% relative humidity) for 24 h. The developed cells then treated with varying concentration (10-80 µg/mL) of optimized formulations (F-1, F-2 and F-3), pure drug CAP, standard drug Adriamycin®. After adding the experimental drugs the cells were adhered *in situ* using 50 µL of 10% trichloroacetic acid and incubated for 60 min at 4°C. Afterwards, cells were stained by pouring 50 µL SRB dye and 1% acetic acid into each well plate. The plates were air dried and unbound dye was removed by washing through 1% acetic acid. Finally,all samples were diluted with 200 µL of 10 mM unbuffered tris-base solution and optical density (OD) was observed at 540 nm on microplate (Bio-rad, Elisa plate reader). The result of % cell growth was expressed in terms of GI_{50} that means 50% cell growth inhibition at the minimum concentration. Optical density was calculated using the following equation[Chaurasia et al., 2016]

$$\%\ \text{control of cell growth} = \frac{\text{OD of control group} - \text{OD of test group}}{\text{OD of control group}} \times 100$$

4.3.5.10 Acute oral toxicity and histopathology

An acute oral toxicity study was performed by following the guidelines of Organization of Economic Co-operation and Development (OECD) on non-pregnant five-week old female Swiss albino mice procured from Institute of Medical Science, B.H.U., Varanasi, India. The study was done by dividing the animals into two groups, normal control (Group I) and optimized formulations (F-1, F-2 and F-3) (Group II) comprising five mice in each. Prior to the experiment, animals were sheltered in polypropylene cage supplemented with pelleted food and distilled water under standard condition. Toxicity examination was carried out by oral administration of 2000 mg/Kg of the formulation batch (F-1, F-2 and F-3) initially to the first animal through a stomach tube and kept under supervision. After survival of the first

animal feeded with the formulation rest of the animals were also administered with the same above mentioned dose and kept under observation for 14 days. On 15th day all animals were sacrificed and their major organs for instance liver, kidney, heart, and spleen were removed, rinsed with saline solution and preserved in 10% v/v formalin solution and fixed in paraffin blocks. The prepared blocks were sectioned (thickness 5 µm), and stained using methylene blue dye and examined microscopically for any histopathological changes [Kaity and Ghosh, 2015, Vijan et al., 2012].

4.3.5.11 Stability study

Stability study of optimized batch (F-1) was performed by packing and storing the formulation in high-density plastic bottles at three storage conditions, 5°C ± 3 °C in refrigerator, 40°C ± 2 °C with 75 ± 5% RH (relative humidity) in controlled oven high temperature and at room temperature 25°C ± 2°Cwith 70 ± 5% RH. The study was conducted for six months by following ICH guidelines Q 1A (R2) [Guideline, 2003]. Finally, to assess the stability of CAP loaded IPN microbeads (F-1) were observed for morphology, particle size and % drug entrapment and shelf life of the optimized batch was evaluated using Minitab-17® software.

4.3.5.12*In vivo* pharmacokinetic study

In vivo pharmacokinetic study was done on male Albino Wistar rats weighing 160-200 g obtained from Institute of Medical Science, BHU, Varanasi. The entire experimentprocedure and the guidelines were approved by the Central Animal Ethical Committee (No.Dean/2017/CAEC/712). Animals were divided into four groups, containing 6 rats in each. Group, I was administered with the pure drugwhile Group II, Group III and Group IV were administered with optimized formulations F-1, F-2, F-3, respectively andwere kept in polypropylene cage provided with nutritious feed and water under standard animal house

conditions.A solution of pure drug and suspensions of optimized formulations (209.4 mg/Kg) were administered orally to the animals of respective groups[Food and Administration, 2005]. Blood samples were collected fromretro-orbital plexus of each animal at specific time intervals of 0, 0.25, 0.5, 1, 4, 6, 8, 10, and 12 h into heparinized tubes. Tubeswere centrifuged at 4000 rpm for 20 min at 4°C to collect the plasma. The extraction of drug from the collected plasma was done by adding methanol and further, centrifuging it at 13000 rpm for 5 min at 4°C. Finally, determination of plasma drug concentration was done on HPLC (Shimdzu corporation, Kyoto, Japan) attached with UV –Visible detector. The detection of drug was done at 232 nm using C- 18 reverse phase column. The samples were injected into the column at a flow rate of 1 mL/min comprising mobile phase as a mixture of methanol and phosphate buffer (pH 6.8) 70:30ratios. Estimation of different pharmacokinetic parameters, anarea under the curve $(AUC)^{t}_{0}$, mean residence time (MRT), elimination half-life ($t_{1/2}$), maximum plasma drug concentration (C_{max}) and time to reach C_{max} (T_{max}) were done by using Kinetica 5.1^{TM} software. The parameters were compared statistically by applying student's t-test and data with $p< 0.05$ were considered significantly different.

4.3.5.13*In vivo* antitumor activity

The antitumor activity of optimized formulationswas studied in Balb/c mice [Fan et al., 2016]. For the activity, mice of either sex weighing approximately 18-20 g were used and divided into groups named as control, optimized formulation (F-1 F-2 and F-3), pure drug and standard. The animals were treated humanely throughout the experiment to reduce the suffering. The mice were inoculated with tumor cells (colon 26-CRC cells). Each group of animals received a specific quantity of dose orally except control that received saline throughout the entire experiment.The experimental animals were feeded with their respective formulation till 30 days. After one month the relative tumor volume (RTV) was estimated for each group by measuring the size of tumor with a digital calliper. Further, the animals were

also checked for survival data and weight loss [Vardhan et al., 2017]. For statistical analysis purpose, GraphPad Prism software was utilized.

PART II

4.4Development of Locust bean gum (LBG) and Sodium alginate (NaAlg) based Aluminum ion (Al^{3+}) cross-linked Interpenetrating Polymeric Network (IPN) loaded with Capecitabine (CAP); (F-2)

4.4.1 Risk assessment study

Risk assessment (RA) involves recognition of hazards and consideration and evaluation of risks related with the exposure to those hazards. (Figure 4.2). RA starts with well-defined risk question. There are three main basic questions that are often helpful during study for instance, what might may go wrong? what is the probability it will go wrong? and what are the consequences or severity? [Food and Administration, 2016]. According to that, all the risks are classified and ranked are shown in Table 4.1. There are several tools for the assessment of risk such as fault tree analysis (FTA), failure mode effective analysis (FMEA), failure mode, effects, and criticality analysis (FMECA), preliminary hazard analysis (PHA) etc. In the present study, FMEA tool has been utilized for the RA study. FMEA involves rating of risks in terms of severity of the risk (S), occurrence of risk (O) and detectability of the risk (D). Finally multiplication all the values of S, O and D to obtain risk priority number (RPN) and determine the area of great concern [Parsana and Patel, 2014] (Table 4.4). In the present study, CMAs and CPPs scoring 10 or >10 were considered as high-risk factors and further studied for FFD. A diagrammatic illustration of RPN scoring is shown in Figure 4.3.

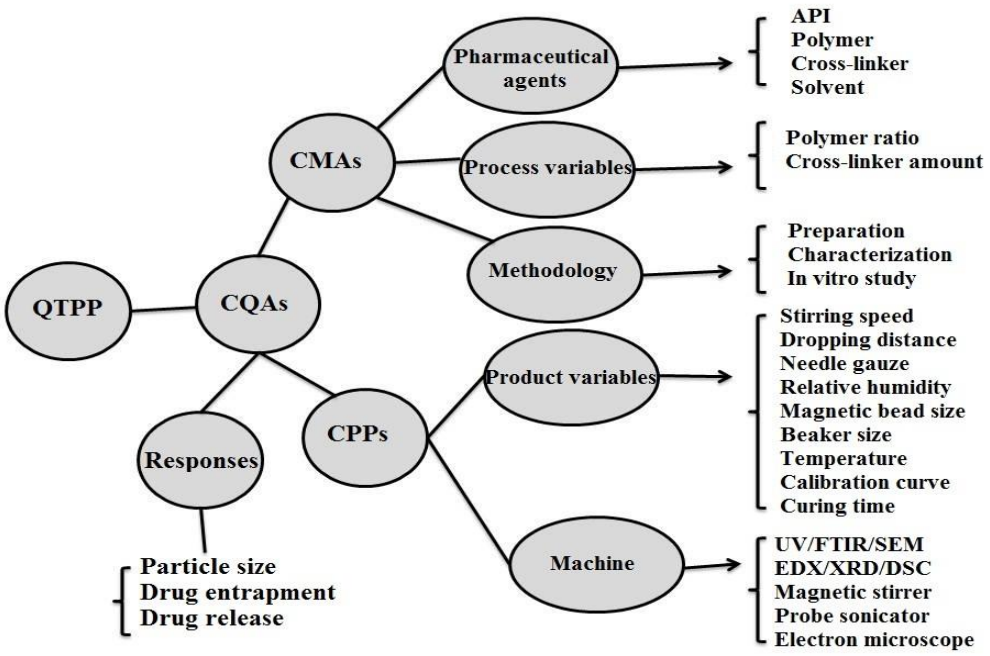

Figure 4.2 Possible CMAs and CPPs influencing the CQAs

4.4.2 Screening: Fractional Factorial Design (FFD)

Screening study has followed the same method as described in the earlier **subsection 4.4.2** The selected coded factors A, B, C, D, and E from RA were subjected into FFD (Table 4.4) and the selection of the factor (that should be taken into major consideration) was done by means of Pareto chart crossing the reference line. The study was performed with the help of Minitab-17® software.

Table 4.4 Assignment of Risk Priority Number

Independent variables (CMAs & CPPs)	S x O x D	RPN
➢ API	2 x 1 x 3	6
➢ Polymer	3 x 1 x 2	6
➢ Cross- linker	3 x1 x 3	9
➢ Solvent	1 x 1 x 3	3
➢ Polymer amount	4 x 2 x 2	16
➢ Cross-linker amount	3 x 2 x 2	12
➢ Preparation	3 x 2 x 1	6
➢ Characterization	4 x 2x 1	8
➢ In vitro study	4 x 2 x 1	8
➢ Stirring speed	2 x 2 x 1	4
➢ Dropping distance	4 x 2 x 2	16

➢ Relative humidity	3 x 2 x 1	6
➢ Magnetic bead size	2 x 2 x 1	4
➢ Beaker size	2 x 1 x 1	2
➢ Temperature	2 x 2 x 2	8
➢ Calibration error	4 x 2 x 1	8
➢ Curing time	4 x 2 x 2	16
➢ Needle gauze	3 x 2 x 2	12
➢ UV/FTIR/SEM/XRD	4 x 2 x 1	8
➢ Magnetic stirrer	2 x 2 x 2	8
➢ Probe sonicator	3 x 2 x 1	6
➢ Electron microscope	3 x 2 x 1	6

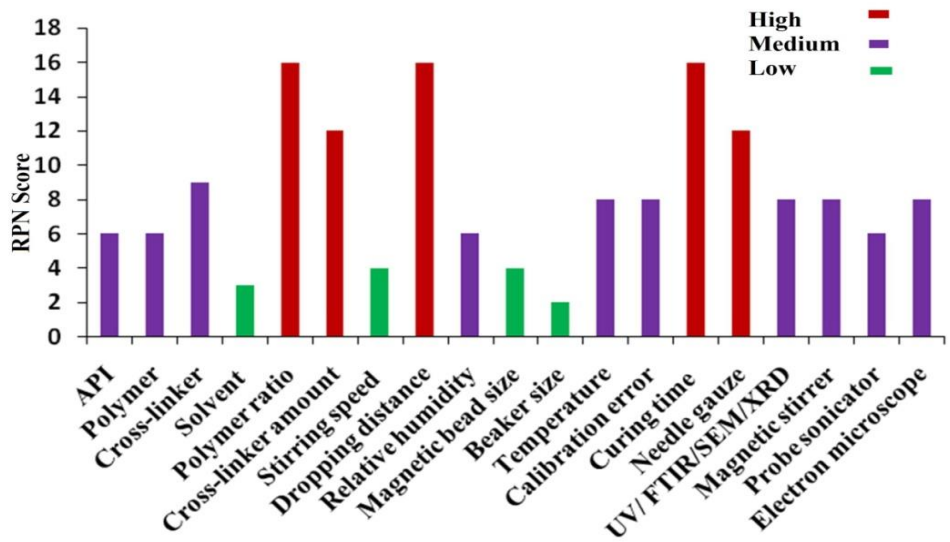

Figure 4.3 Result of RPN score of possible CMAs & CPPs

Table 4.5 FFD of the experiment

Runs	A	B	C	D	E	Y_1 (µm)	Y_2 (%)	Y_3 (%)
1	+	–	–	–	–	510.67	80.34	84.08
2	+	–	+	–	+	515.31	77.19	81.39
3	+	+	–	+	–	496.18	85.51	80.46
4	+	+	+	+	+	500.23	81.60	63.27
5	–	–	–	+	+	354.29	66.26	86.45

6	−	+	−	−	+	341.50	73.59	72.15
7	−	+	+	−	+	338.77	70.13	70.89
8	−	−	+	+	+	360.43	63.60	73.54

A= polymer ratio, B= amount of cross-linker, C= curing time, D= needle gauze, E= dropping distance

4.4.3 Optimization and statistical analysis

After recognition of risks during product development, further design of experiments (DOE) is carried out to enhance the process knowledge [Kan et al., 2014]. DOE links the effect of independent factors with the response and well manages the relationship between CPPs and CQAs [Guideline].The most commonly used DOE is BBD as it is the most convenient method that requires fewer method [Motwani et al., 2008]. The BBD employed in the present study has 3 levels, 3 factors with 3 Centre points. The independent factors selected were amount of polymer (A); amount of cross-linker (B); and curing time (C) whereas the responses selected were particle size (Y_1); % drug release (Y_2) and % drug entrapment (Y_3). For statistical analysis, p-value, regression coefficient (R^2), Fisher's value (F) and lack of fit value, all these value were checked for their suitability to be fit for the model. On the basis to be fit to the model quadratic equations were generated and further optimized batch was selected based on dependent variable constraint and utilized for all *in vitro* and *in vivo* characterizations.

4.4.4Preparation of Al^{3+} cross-linked IPN encapsulating CAP using natural polymers LBG and NaAlg

CAP encapsulated IPN microbeads of biopolymers LBG and NaAlg were prepared by the previously reported method [Upadhyay et al., 2018]. Briefly weighed quantity of CAP (50 mg), and the polymers LBG and NaAlg (1:1 out of 1000 mg total weight of polymers) were stirred completely on magnetic stirrer (IKA RH digital) at 100 rpm. The formed homogenous

viscous solution of drug and polymer was filled into 23 gauze needle and transferred drop-wise to another aqueous media of cross-linker (2%w/v) aluminum chloride (Al^{3+}) with continuous stirring at 50 rpm. The formed beads were cured in the cross-linker solution for 60 min. Finally, the beads were filtered, washed repeatedly, dried at room temperature and stored in a desiccator until further use. A diagrammatic illustration of preparation of IPN microbeads is presented in Figure 4.4.

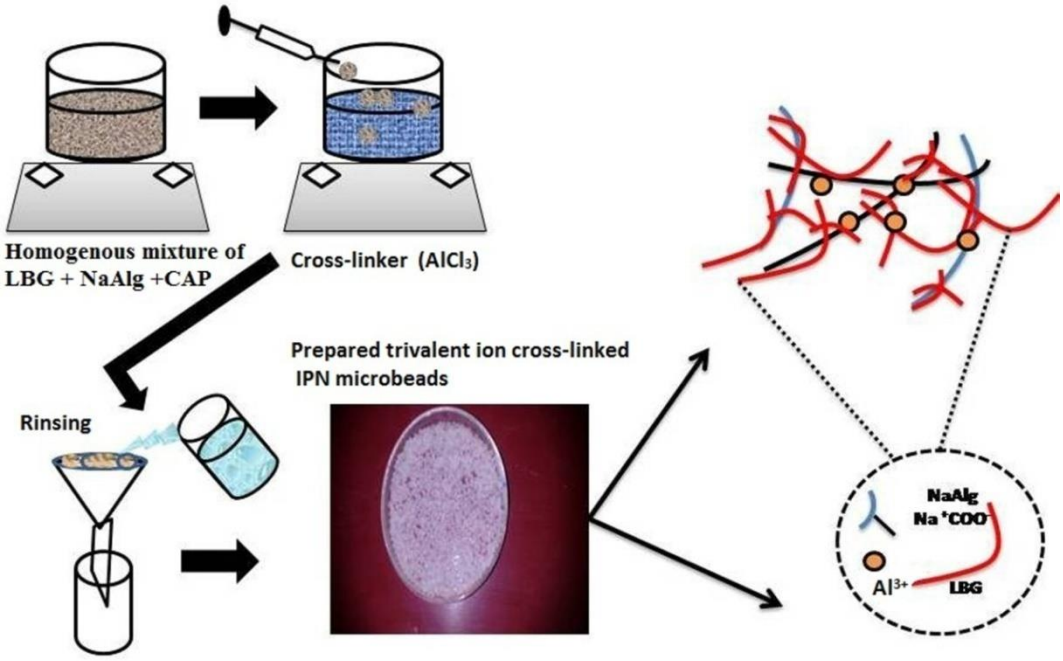

Figure 4.4 Preparation of trivalent ion (Al^{3+}) IPN microbeads

4.4.5 Characterization

4.4.5.1 Determination of particle size

4.4.5.1.1 Optical electron microscopy

Particle size estimation of the formed microbeads was performed in a similar way as discussed in **subsection 4.3.5.1.1**

4.4.5.1.2 Scanning electron microscopy (SEM)

Examination of surface morphology of formed IPN microbeads was done on SEM instrument (Supra 40, Zeiss, Japan) following the same procedure as discussed in **subsection 4.3.5.1.2**

4.4.5.2 Energy Dispersive X-ray (EDX)

EDX of the formed sample was performed on EDX instrument (Ametek, NJ). Detection of the elements and their percentage estimation was done by following the same method as discussed in **subsection4.3.5.2**

4.4.5.3 Fourier Transform Infra-Red spectroscopy (FTIR)

Physicochemical compatibility study of LBG, NaAlg, CAP, physical mixture and prepared formulation was done on FTIR (Shimadzu, 8400S, Japan) as discussed in **subsection 4.3.5.3**

4.4.5.4 X-ray Diffraction (XRD)

XRD spectra of LBG, NaAlg, physical mixture and CAP carrying IPN microbeads were examined on instrument X-ray diffractometer (Rigaku, Japan) as mentioned in **subsection 4.3.5.4**

4.4.5.5 Differential scanning calorimetry (DSC)

DSC was performed as discussed in **subsection 4.3.5.5**

4.4.5.6 Drug entrapment (%)

Drug entrapment was estimated by the same procedure as discussed in **subsection 4.3.5.6**

4.4.5.7 Swelling study

Swelling study was performed using the same gravimetric method as discussed in **subsection 4.3.5.7**

4.4.5.8 In vitro drug release study

Performed following same method as mentioned in **subsection 4.3.5.8**

4.4.5.9*In vitro* cell cytotoxicity study

Same method was followed as mentioned in **subsection4.3.5.9**

4.4.5.10 Acute oral toxicity and histopathology

The study was performed by following the similar procedure as discussed in **subsection4.3.5.10**

4.4.5.11 Stability study

The study was done by following the same method as described in **subsection 4.3.5.11**

4.4.5.12*In vivo* pharmacokinetic study

The study was conducted by following the same procedure as discussed in **subsection4.3.5.12**

4.4.5.13*In vivo* antitumor activity

The study was performed as described in **subsection4.3.5.13**

PART III

4.5Development of Locust bean gum (LBG) and Sodium alginate (NaAlg) based Aluminum ion (Al^{3+}) cross-linked buoyant and mucoadhesive Interpenetrating Polymeric Network (IPN) loaded with Capecitabine (CAP); (F-3)

4.5.1 Risk assessment study

Risk assessment study was performed to identify CMAs and CPPs during development of buoyant IPN microbeads that are likely to affect CQAs of the formulation. A cause and effect diagram i.e. Ishikawa fish bone diagram (Figure 4.5) was also constructed to build the potential relationship among the variables with the help of Minitab-17® software. Further,

estimation of high risks by means of risk estimation matrix (Table 4.7). The rank orders falling in the range between 1 and 20 were assigned to the CMAs or CPPs for severity, occurrence and detectability to estimate the RPN score. (Table 4.6) Finally the factors with score 10 or > 10 were selected and subjected to another screening study Fractional Factorial Design (Table 4.8).

Table 4.6 Estimation of Risk Priority Number

Risk area	S x O x D	RPN
CMAs		
Active pharmaceutical agents		
➢ API	2 x 1 x 3	6
➢ Polymer	3 x 1 x 2	6
➢ Cross- linker	3 x1 x 3	9
➢ Solvent	1 x 1 x 3	3
Process variable		
➢ Polymer amount	4 x 2 x 2	16
➢ Cross-linker amount ➢ Effervescent agent amount	3 x 2 x 2 4 x 3 x 2	12 24
Methodology		
➢ Preparation	3 x 2 x 1	6
➢ Characterization	4 x 2x 1	8
➢ In vitro study	4 x 2 x 1	8
CPPs		
Product variable		
➢ Stirring speed	2 x 2 x 1	4
➢ Dropping distance	4 x 2 x 2	16
➢ Relative humidity	3 x 2 x 1	6
➢ Magnetic bead size	2 x 2 x 1	4
➢ Beaker size	2 x 1 x 1	2
➢ Temperature	2 x 2 x 2	8
➢ Calibration error	4 x 2 x 1	8
➢ Curing time	4 x 2 x 2	16
➢ Needle gauze	3 x 2 x 2	12
Machine		
➢ UV/FTIR/SEM/XRD	4 x 2 x 1	8
➢ Magnetic stirrer	2 x 2 x 2	8
➢ Probe sonicator	3 x 2 x 1	6
➢ Electron microscope	3 x 2 x 1	6

Table 4.7 Risk estimation matrix

CQAs	API	Polyera mount	Crosslinker type	Solvent	Effervescent agent amount	Stirring speed	Crosslinker amount	Dropping distance	Needle gauze	Relative humidity	Magnetic bead size
Particle size	Med	High	Med	Low	High	Med	High	High	High	Med	Low
% Drug entrapment	Med	High	High	Low	Low	Med	High	High	High	Med	Low
% Buoyancy	Med	High	Low	Low	High	Low	Low	Low	Low	Low	Low

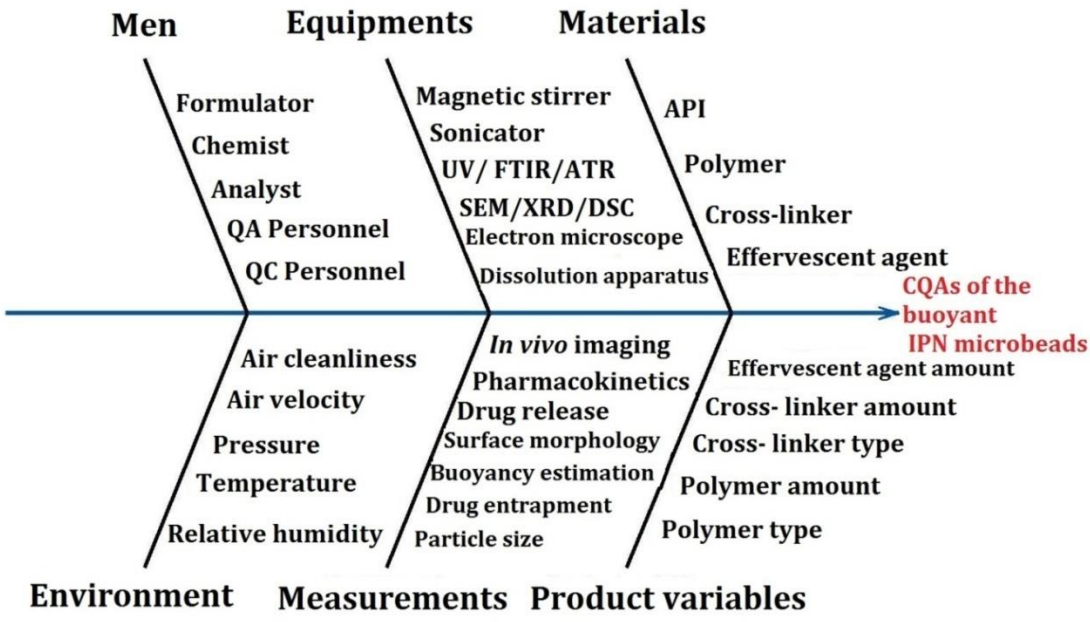

Figure 4.5 Ishikawa fishbone diagram: Cause and Effect relationship

4.5.2 Screening study: FFD

Screening study followed the same method as described in the earlier **subsection 4.3.2 and subsection 4.4.2** The highest RPN scored coded factors A, B, C, D, and E from FMEA were subjected to FFD (Table 4.6). The study was performed with the help of Minitab-17® software.

Table 4.8 Fractional Factorial design study

Runs	A	B	C	D	E	Y_1(µm)	Y_2(%)	Y_3(%)
1	−	−	−	+	+	500.54	61.45	59.11
2	+	+	+	+	+	367.09	95.14	86.44
3	+	−	−	−	−	695.67	83.91	77.13
4	+	−	+	−	+	736.44	87.44	86.20
5	+	+	−	+	−	359.19	98.54	79.65
6	−	−	+	+	−	520.32	59.12	62.04
7	−	+	−	−	+	456.96	76.33	73.31
8	−	+	+	−	−	440.78	72.28	60.54

A= polymer ratio, B= amount of cross-linker, C= amount of $NaHCO_3$, D= needle gauze, E= dropping distance

4.5.3 Optimization and statistical analysis

The design of the experiment and its statistical validation was done by employingDesign Expert® software Version 11. BBD was used for the optimization of the formulation parameters involving study of three independent factors polymer ratio (A), amount of cross-linker (B) and amount of $NaHCO_3$ (C) on particle size (Y_1), drug entrapment (Y_2) and buoyancy (Y_3). All the responses were fitted into linear, quadratic and two-factor interaction model and evaluated by the statistical significance of the coefficient and R^2 values. Based on the selected model, polynomial equations were generated for each response and based on the dependent variable constraints an optimized batch was selected [Singh et al., 2016]. Further,

for all the characterization techniques and in vitro drug release study only the optimized batch was considered.

4.5.4 Preparation of buoyant Al^{3+} cross-linked IPN encapsulating CAP using natural polymers LBG and NaAlg

Bio polymeric IPN microbeads of LBG and NaAlg containing CAP were prepared by ionotropic gelation method following the similar method as discussed in **subsection4.4.4**with slight modification**.** Briefly, CAP (50mg), blend of LBG and NaAlg (1:1 out of 1000mg total weight of polymers) and sodium bicarbonate (100mg), a gas generating agent ($NaHCO_3$) were initially dispersed in aqueous media and stirred well on magnetic stirrer (IKA® RH digital) (Figure 4.6A) The formed homogenous solution was then added drop-wise through 23G into another solution of trivalent aluminum chloride ($Al^{3+}/AlCl_3$; 2% w/v) containing hydrochloric acid solution (1%v/v). The formed suspended IPN microbeads then left in the solution while the stirring was continued for approximately 20 min in order to enhance the mechanical strength of the microbeads. For a system to float, liberation of carbon-di-oxide (CO_2) occurs that can be released only in the presence of acidic media (HCl). The scheme of the release of CO_2 is shown in Figure 4.6B. The prepared buoyant IPN microbeadswere finally collected, washed with double distilled water and then dried at room temperature.

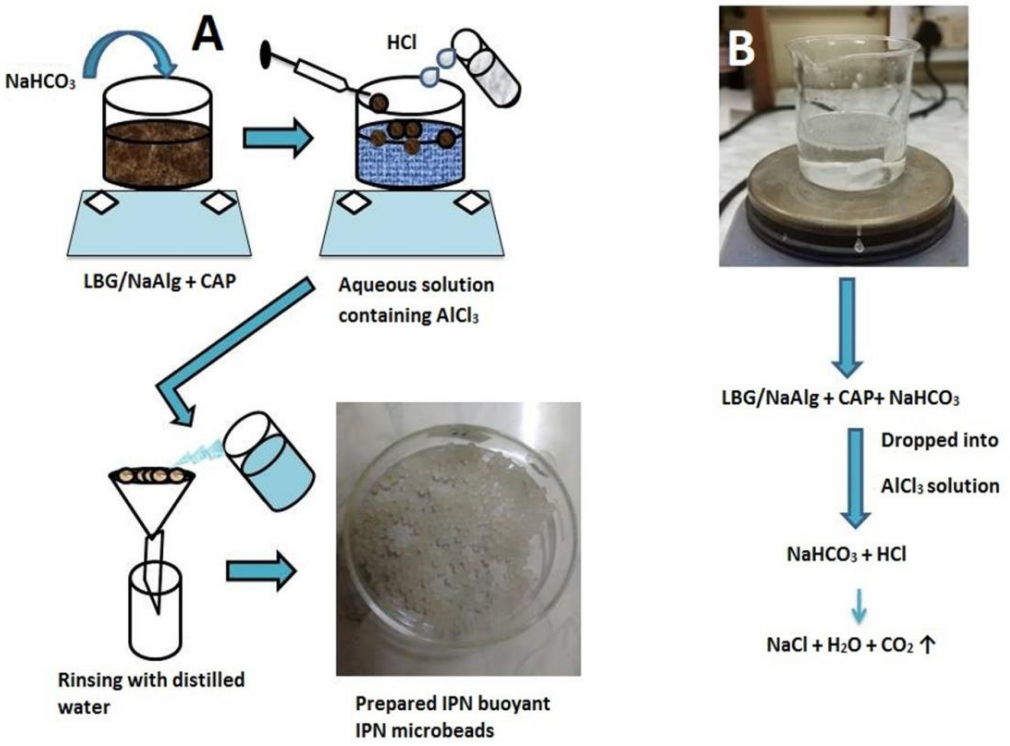

Figure 4.6 (A) Step-wise preparation of IPN buoyant IPN microbeads; (B) Mechanism of buoyancy

4.5.5 Characterization

4.5.5.1 Determination of particle size

4.5.5.1.1 Optical electron microscopy

Determination of particle size was performed in a similar way as discussed in **subsections4.3.5.1.1**

4.5.5.1.2 Scanning electron microscopy (SEM)

The same procedure was followed for determination of surface morphology as mentioned in **subsections 4.3.1.5.1.2**

4.5.5.2 Energy Dispersive X-ray (EDX)

The same procedure was followed as discussed in **subsections4.3.5.2**

4.5.5.3 Fourier Transform Infra-Red spectroscopy (FTIR)

A similar method was followed as mentioned in **subsections4.3.5.3**

4.5.5.4 X-ray Diffraction (XRD)

The same method was followed as mentioned in **subsections4.3.5.4**

4.5.5.5 Differential scanning calorimetry (DSC)

A similar procedure was followed as discussed in **subsections4.3.5.5**

4.5.5.6 Drug entrapment (%)

A similar procedure was used for drug entrapment estimation as discussed in **subsections 4.3.5.6**

4.5.5.7 Swelling study

The study was performed using the similar method as discussed in **subsections4.3.5.7**

4.5.5.8 Estimation of bead size and density

Bead size measurement was estimated by using digital slide vernier calipers (ABSOLUTE DIGIMATIC, Mitutoyo Corp., Japan). Approximately 20 beads were selected randomly from each formulation batch and measured individually for their size measurement. The density of each microbeads was calculated with the help of the following equation:

$$\rho = \frac{M}{V} \quad \text{and} \quad V = \frac{4}{3}\pi r^3$$

where ρ= density (g/cm^3), M= weight (g), V= volume (cm^3) and r= radius (cm)

4.5.5.9*In vitro* buoyancy study

The floating ability of the IPN microbeads was determined by following the earlier reported method[Singh et al., 2015]. Briefly, 350 mg of IPN microbeads were placed in 100 mL of SGF. Due to buoyant in nature, the beads appeared initially on the surface of the media. Afterwards, the media containing formulation was stirred by a magnetic stirrer at 100 rpm for about 12 h. The time taken by the microbeads to emerge at the water surface and the time the formulation to remain float on the surface of the media were evaluated in terms of floating lag time (FLT) and total floating time (TFT) respectively and % buoyancy was calculated using the following equation:

$$\text{Buoyancy (\%)} = \frac{weight\ of\ floated\ microbeads}{weight\ of\ floated\ microbeads - weight\ of\ settled\ microbeads} \times 100$$

4.5.5.10 *Ex-vivo* mucoadhesion

The mucoadhesive property of the optimized formulation was performed by wash off method [Nayak et al., 2014, Pal and Nayak, 2011]. The fresh excised piece of the model membrane intestinal mucosa of the goat (2x2cm), collected from the local slaughterhouse was rinsed with physiological saline to remove off the debris adhered to the membrane. Briefly, 50 IPN microbeads were scattered on the membrane and then it was adjusted and tied onto burette stand clamp to the nearest distance in a such a way so that at every up and down movement of the USP tablet disintegration apparatus (Electrolab, Mumbai, India) the mucosal membrane remain in contact with the test fluid.At the end of 5h and at periodical time interval of 1 h the number of microbeads adhered to the membrane was counted. The test was carried out at both gastric pH (0.1 N HCl, pH 1.2) and intestinal pH (phosphate buffer, pH 6.8) and was calculated using the formula

$$Mucoadhesion\ (\%) = \frac{Microbeadstaken - nonadherentmicrobeads}{Microbeadstaken} \times 100$$

4.5.5.11*In vitro* drug release

The *in vitro* drug release of optimized IPN microbeads was done using USP type II dissolution apparatus (Electrolab, Mumbai, India). An equivalent weight of microbeads corresponding to 500 mg of CAP was placed in 900 mL of SGF (pH 1.2) for 8 h followed by pH 6.8 until complete drug release. The dissolution apparatus was maintained at 37±0.5°C and operated at 50 rpm. The aliquot of 5 mL were withdrawn at regular time intervals and analyzed spectrophotometrically (Shimadzu 1800, Japan) at 239 nm.

4.5.5.12*In vitro* cell cytotoxicity study

The same method was followed as mentioned in **subsection4.3.5.9**

4.5.5.13Acute oral toxicity and histopathology

The study was performed by following a similar procedure as discussed in **subsection4.3.5.10**

4.5.5.14 Stability study

The study was performed by following the same procedure as described in **subsection4.3.5.11**

4.5.5.15*In vivo* pharmacokinetic study

The study was conducted by following the same procedure as discussed in **subsection4.3.5.12**

4.5.5.16*In vivo* antitumor activity

The study was performed by following the same procedure as described in **subsection 4.3.5.13**

4.5.5.17*In vivo*gastroretention study: Gamma (γ) scintigraphy study

The study was performed to determine the location and extent of transit time through the gastrointestinal tract of the optimized gastroretentive IPN microbeads after its administration

to the mice through the oral route. The experiment was conducted as performed previously [Md et al., 2011]. For the study, the Al^{3+}cross-linked buoyant IPN microbeads were initially prepared as discussed in **subsection 4.5.4** Afterwards, the formed beads were radiolabeled with ^{99m}TC (Technetium) following the procedure described elsewhere. Briefly, to 2 mci/mL (microcurie)^{99m}TC solution, 0.2 mL of stannous chloride solution (1mg/mL) was added and mixed homogenously. Both the aqueous solutions were prepared in nitrogen purged water. The pH of the solution was maintained at 7.5 with 0.5 M sodium bicarbonate solution. Finally, to above-formed radiolabel containing solution approximately weighed 5 mg of formed trivalent ion cross-linked buoyant IPN microbeads were added and incubated for 15 min at room temperature [Halder et al., 2008].The imaging was done by administering 1 mL of preparation orally to themice of 18-20 g. The images were captured after 0.5, 2, 4 and 6 h of dosing with large field view γ camera (Varicam, Panasonic, Japan) associated with high-resolution parallel-hole collimator and interfaced to a computer.[Md et al., 2011].