## 2. Experimental

### 2.1. Materials

Chitosan, MW 222 kDa, degree of deacetylation (DDA) 82% was obtained from Sigma-Aldrich, USA. The DDA of chitosan was determined by potentiometric titration[27] and its molecular weight was determined using gel permeation chromatography (Waters, USA). Sodium alginate (β-D-mannuronic acid (M)/α-L-guluronic acid (G) content 64.5%/35.5%), quercetin and streptozotocin (STZ) were also purchased from Sigma-Aldrich, USA. White crystalline potassium bromide (KBr) was obtained from Merck (India). Succinic anhydride and dimethyl sulphoxide (DMSO) were purchased from Sisco Research Laboratories (SRL), India. The cholesterol and triglyceride kits were purchased from Span Diagnostics Ltd. (Mumbai, India). All other reagents were of analytical grade and were used directly with further purification.

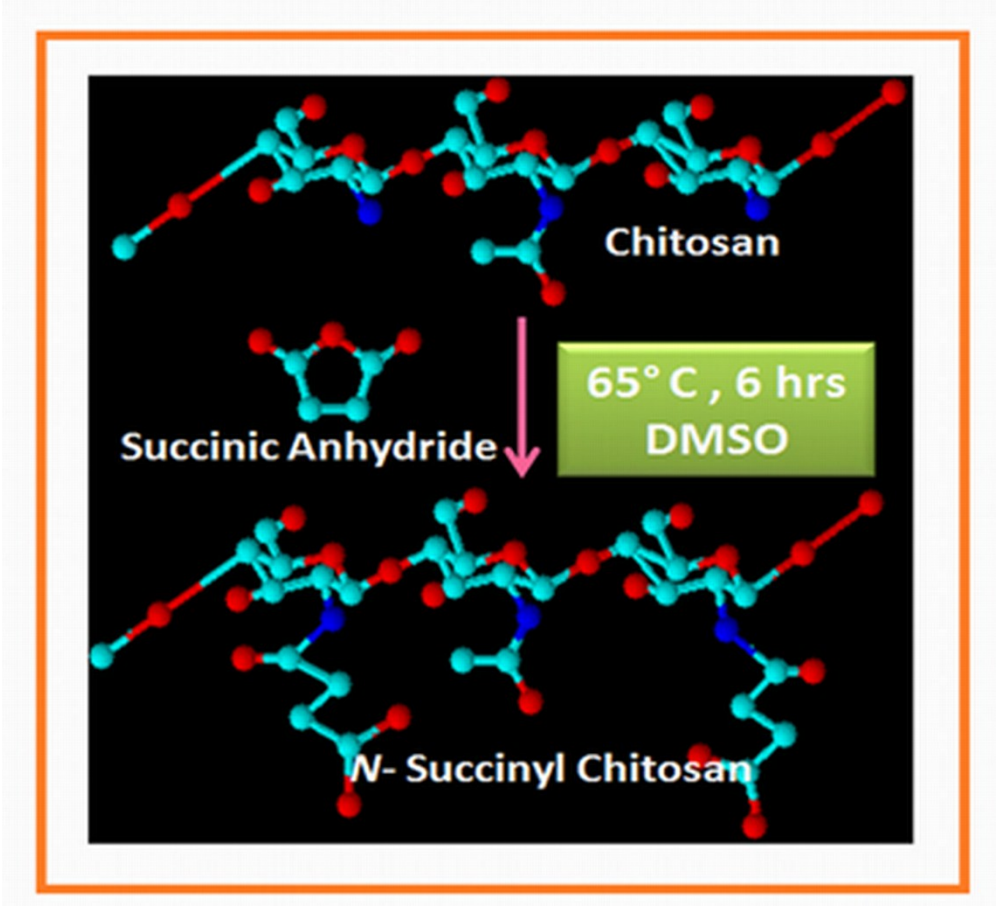
### 2.2. Animals

Male Wistar rats weighing 100–120 g (M/s Chakraborty Enterprise, Kolkata, India) were acclimatized under a control laboratory environment (temperature 24±2ºC, relative humidity 60±5%, and 12/12 hr light dark cycle) with balanced diet (standard pellet food procured from Hindustan Uniliver, India) and water *ad libitum* for about 2 weeks prior to initiate the experiments. All animal experiments in this study were approved by animal ethics committee (IAEC) of University of Calcutta (Registration No. 935/c/06/CPCSEA, 30.06.2009) as per guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

### 2.3. Synthesis of *N* -succinyl chitosan (NSC)

Water soluble chitosan derivative *N*-succinyl chitosan (NSC) was prepared by ring-opening reaction using succinic anhydride in dimethyl sulfoxide following our previous report with little modifications.[28] In brief, chitosan (2.0g) was suspended in 40 mL DMSO containing

succinic anhydride and the reaction was further carried out at ~65°C for 6 hr. The reaction mixture was filtered and the precipitate was dispersed in ethanol and kept at room temperature for an hour. The pH of the dispersion was adjusted to 10-12 using 1M NaOH and then filtered. The final product was purified by dissolving it in 90 mL of distilled water and reprecipitated by 270 mL acetone. The precipitate was filtered and washed again with ethanol followed by acetone and finally vacuum dried at 50°C to get the final product.




**Reaction of chitosan and succinic anhydride to produce pH sensitive, water soluble N-succinyl chitosan (NSC)**

### 2.4. Determination of degree of substitution of NSC

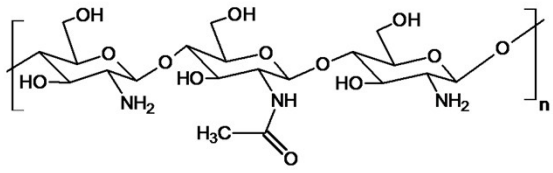
The potentiometric titration method was used to measure the degree of substitution (DS) of the prepared NCS according to the previous report.[28]

## 2.5. Reaction energetics study of chitosan and succinic anhydride to produce *N*-succinyl chitosan

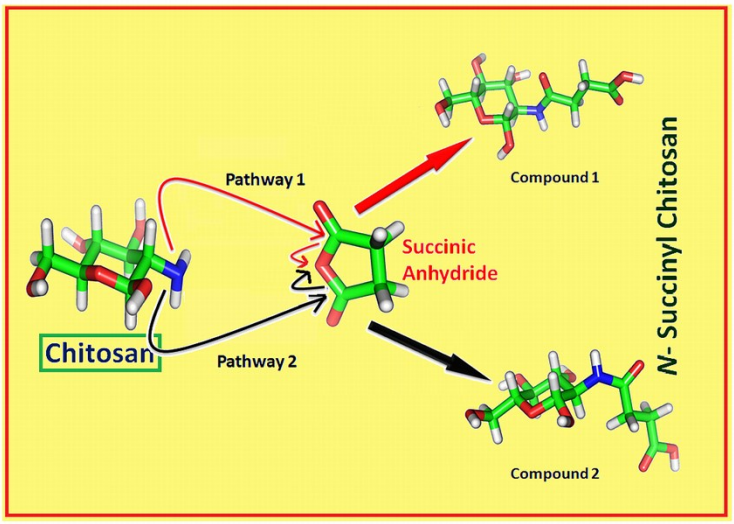
Reaction energetics between D-glucosamine and succinic anhydride to produce succinyl chitosan was explored using quantum mechanical (QM) calculations in vacuo using HYPERCHEM 8.0. During the calculation, the monomeric unit of chitosan (D-glucosamine) and succinyl chitosan were considered for ease of computation. All the reactants and products were fully minimised using Polak–Ribiere conjugate gradient with an RMS gradient of 0.001 kcal $A^{-1}mol^{-1}$ by using two semi-empirical methods, AM1 and RM1 implemented in HYPERCHEM 8.0. The energy change involved in each reaction pathway was calculated as:

$$\Delta E = E_{compound} - (E_{D\text{-}glucosamine} + E_{succinic\ anhydride}) \qquad (1)$$

where $E_{compound}$, $E_{D\text{-}glucosamine}$ and $E_{succinic\ anhydride}$ are the energies of the product compounds (i.e., compound 1 and 2) and D-glucosamine and succinic anhydride, respectively.




Chitosan




**Schematic representations of the two possible reaction pathways considered in the molecular modelling study are shown. Pathway 1: Succinic anhydride attacks through above the plane of chitosan monomer unit; Pathway 2: Succinic anhydride attacks through below the plane of chitosan.**

**2.6. Quercetin (QUE) loaded microparticles preparation and characterization**

*N*-succinyl chitosan and sodium alginate blend hydrogel microparticles were evaluated in oral quercetin delivery and they were prepared by dropping the homogeneous aqueous mixture of *N*-succinyl chitosan and sodium alginate (with distinct NSC-to-alginate weight ratios of 0:1, 2:1, 1:1, 0.5:1, 0.25:1, 1:0) in 2% calcium chloride solution using a 21 gauge needle.[29] A stock solution of NSC in water (3%, w/v) was prepared by dissolving 0.6 g of NSC in 20 mL deionized water under continuous stirring for 12-15 hr at room temperature. Subsequently, appropriate amounts of sodium alginate, as per the NSC-to-alginate weight ratios, were added to the stock solution. The microparticles were prepared by extrusion technique. We have prepared the microspheres by dropping the bubble-free dispersion through a disposable syringe (with a nozzle of 0.5 mm inner diameter) into 20 ml of a gently agitated solution of the crosslinking agent (2% $CaCl_2$). The dropping rate was 30 microparticles / min. The falling distance was 5 cm. The gelled microspheres were separated, unless otherwise noted, after a reaction time of 30 mins, washed with deionized water and then air dried for 24 hr. The prepared microparticles were allowed to crosslink for 30 mins at room temperature in the 2% calcium chloride solution**.** The microparticles were washed with distilled water thrice and dried under vacuum, at 37°C temperature overnight.

Quercetin solution (1mg/mL) was added to the polymeric mixture to prepared QUE loaded microparticles following the same process above.

Fourier transform infrared (FT-IR) spectroscopic analysis was carried out with Bruker Alpha ATR FT-IR spectrometer (Model: Alpha –E). All the samples were mixed uniformly with potassium bromide at 1:10 weight ratio and KBr pellets were prepared using 10 ton hydraulic pressure for 10 min at room temperature. Then, FT-IR spectra of the pellets were recorded within the frequency range of 4000–500 $cm^{-1}$ for 42 consecutive scans.

X-ray diffraction spectrometry of the polymeric samples in the powder form was examined using a wide angle X-ray scattering diffractometer (Pananalytical X-Ray Diffractometer, model-X'pert Powder) employing Cu $K_\alpha$ filtered radiation ($\lambda$= 1.54060). The XRD scan rate was fixed at 1°$min^{-1}$ and the step size was 0.04°. The accelerating voltage of 40K and 30mA current were used during the scanning process.

The morphology and shape of the blank and quercetin loaded NCS-ALG microparticles were observed under a scanning electron microscope (EVO-18; Carl Zeiss, Oberkochen, Germany). Prior to imaging, the dried microparticles were taped on SEM stub and sputter coated with a thin layer of gold using a Hitachi sputter coater (Model- E1010 Ion sputter) under vacuum to neutralize the charging effects before scanning under SEM at an acceleration voltage of 15kV.

**2.7. Swelling and deswelling study of the microparticles**

In order to have a clear idea about pH sensitive swelling of these microparticles, swelling - deswelling behaviour of those formulations was observed in different pH solutions (simulated gastric fluid, NaCl 2.0 g, hydrochloric acid 7 mL, $H_2O$ up to 1 litre, pH adjusted to 1.2 and simulated intestinal fluid, NaCl 9 g, $Na_2HPO_4$ 0.072 g, $NaH_2PO_4$ 0.035 g, $H_2O$ up to 1 litre, pH adjusted to 7.4) corresponding to the gastrointestinal tract (GI) pH in accordance to our earlier reports with slight modifications.[29] The swelling ratio ($Q_s$) was calculated using the following formula:[29]

$$Q_S = \frac{W_S - W_d}{W_d} \qquad (2)$$

Where $W_s$ and $W_d$ are the weight of the swollen and dry microparticles, respectively.

## 2.8. pH dependent interactions modeling between alginate and carboxypropionylated chitosan

The lowest energy succinyl chitosan monomer unit obtained from the molecular modelling study of chitosan and succinic anhydride to produce succinyl chitosan, mentioned in section 2.5 (compound 1) was used in this study. Structure of alginate was drawn using the molecular builder interface of HYPERCHEM 8.0 and fully minimised using Polak–Ribiere conjugate gradient with an RMS gradient of 0.001 kcal $A^{-1}mol^{-1}$ by using both RM1 and AM1 semi-empirical method. To mimic the low pH condition, both the carboxylic acid groups of succinyl chitosan unit and alginate were modelled as COOH and bring both the structures close to each other and stochastically optimised using both AM1 and RM1 semi-empirical method to obtain a possible succinyl chitosan-alginate complex with an RMS gradient of 0.005 kcal $A^{-1}mol^{-1}$.

To mimic pH 7.4, both the carboxylic acids groups from succinyl chitosan and alginate, respectively were modelled as $COO^-$ in the optimised structure of the succinyl chitosan-alginate complex and then the structure was further optimised using AM1 and RM1 semi-empirical method with an RMS gradient of 0.005 kcal $A^{-1}mol^{-1}$.

The energy of complex formation was calculated as:

$$\Delta E = E_{\text{Alginate-N-Succenyl chitosan complex}} - (E_{\text{Alginate}} + E_{\text{N-Succenyl chitosan}}) \quad (3)$$

where $E_{\text{Alginate-N-Succenyl chitosan complex}}$, $E_{\text{Alginate}}$ and $E_{\text{N-Succenyl chitosan}}$ are the energies of the Alginate-N-Succenyl chitosan complex, Alginate and N-Succenyl chitosan, respectively.

## 2.9. Estimation of quercetin loading and quercetin encapsulation efficiency

In order to determine the drug load and encapsulation efficiency, microparticles equivalent to 5 mg of QUE payload were dispersed in 5 mL of simulated intestinal fluid (SIF, NaCl 9 g, $Na_2HPO_4$ 0.072 g, $NaH_2PO_4$ 0.035 g, $H_2O$ up to 1 litre, pH 7.4), and were allowed to swell completely for 6 hr at 37°C. Swollen microparticles were crushed with glass mortar pestle

and centrifuged at 10, 000 rpm to get actual encapsulated quercetin within the microparticles. The clear supernatant was analyzed for quercetin content using UV spectrophotometer at 375 nm.[30] The following formulae were used to calculate the loading and entrapment efficiency of quercetin.[29] All the experiments were run in triplicate.

$$\% \text{ Drug loading } = \frac{\text{Amount of drug in microparticles}}{\text{Amount of microparticles}} \times 100 \qquad (4)$$

$$\% \text{ Encapsulation efficiency} = \frac{\text{Actual loading}}{\text{Theoretical loading}} \times 100 \qquad (5)$$

**2.10. Quercetin release profile and Mathematical modelling of release kinetics**

For drug release experiment, polymeric microparticles equivalent to 10 mg of QUE payload were dispersed in 10 mL of SGF (simulated gastric fluid, NaCl 2.0 g, hydrochloric acid 7 mL, $H_2O$ up to 1 litre, pH adjusted to 1.2 ), and SIF (simulated intestinal fluid, NaCl 9 g, $Na_2HPO_4$ 0.072 g, $NaH_2PO_4$ 0.035 g, $H_2O$ up to 1 litre, pH adjusted to 7.4), each and were transferred into dialysis bags with 12.4 KD MW cut off and was placed in beakers containing 100 mL of buffer solutions respectively, on a shaker bath (BS-06, Medline scientific) at 37.0±0.5 ºC, 50 rpm. Sodium lauryl sulfate (SLS) (3% w/v) has been dissolved in the buffer solution to maintain the sink condition as it is well known that quercetin is sparingly soluble in aqueous buffers. At predetermined time intervals, 10 mL of buffer solution was collected for analysis and replaced with fresh buffer to maintain proper sink conditions. The concentration of the released quercetin at definite time intervals was estimated from absorbance at 375 nm from the collected release buffer aliquots. To deduce QUE release mechanism from the microparticles, the *in vitro* release study data were analysed by Ritger-Peppas model .[31,32]

$$\frac{M_t}{M_\infty} = Kt^n \ldots\ldots\ldots\ldots\ldots\ldots (6)$$

where, $M_t$ and $M_\infty$ are the absolute amount of QUE released at time (t) and infinite time, respectively; the values of K (constant showing structural and geometric characteristic of the device) and *n* (release exponent reflecting the diffusion mechanism) are calculated accordingly to determine the release mechanism.

### 2.11. *Ex vivo* mucoadhesion study

*Ex vivo* mucoadhesive nature of the microparticles was observed with freshly excised rat intestinal lumen following a previously described method with slight modifications.[32]

### 2.12. *In vivo* pharmacological response

The experimental animals were weighed and basal blood glucose level was measured using Accucheck glucometer, before induction of diabetes. Diabetic animal model was prepared by single interperitoneal injection of STZ at a dose of 50 mg/kg b.w in freshly prepared citrate buffer (0.1 M pH 4.0) to overnight fasted rats.[33] Treated animals were given standard food pellet and 5% glucose solution *ad libitum*, for about 2-3 days to overcome the hypoglycaemic shock. Afterwards the glucose solution was replaced with water *ad libitum*. The control animal group received the buffer alone. The fasting glucose level was measured by taking blood from the tail vein. Rats with a blood glucose level above 250 mg/dL were considered as stable hyperglycemic and were used for further experiments.

Animals were randomly divided into four groups each containing 6 animals (n=6)

NC – Normal control received normal saline solution orally.

DC – STZ treated rats served as diabetic subjects.

QT – STZ induced diabetic rats treated with single oral dose of free quercetin (600 mg/kg b.w).

QMT – STZ induced diabetic rats treated with single dose oral of quercetin loaded microparticles (600 mg/kg b.w).

Fasting blood glucose (FBG) level was estimated in blood samples (0.02 mL) collected from the tail vein of each animal at 2 hr interval for 24 hr. The blood glucose was measured using a glucometer (Accucheck) with maximum measuring capacity of ~ 600 mg/dL.

**2.13. Toxicity study**

Acute toxicity study was carried out in order to evaluate the systemic toxic effects of quercetin loaded polymeric microparticles on normal male Wistar rats (weighing approximately 100-120 g). Several routine biochemical, haematological and histological parameters were studied. Animals were fed with a standard diet and had free access to water during the entire course of experiment. Male Wistar rats were divided into following groups having 6 rats in each group (n=6).

NC group - Normal control rats treated with normal saline orally.

$NT_{BM}$ group - Rats treated with blank microparticles (600 mg/kg b.w)

$NT_{QM}$ - Rats treated with quercetin loaded microparticles (600 mg/kg b.w)

Fasting blood glucose levels were checked from blood samples extracted from tail vein of treated rats. Serum was separated from the unheparinised clotted blood by centrifugation at 2500 rpm for 10 mins at 4°C. Cholesterol and triglycerides were measured in the serum samples using standard commercial assay kits following the manufacturer's protocol. Different hepatic damage indices like serum alanine transaminase (ALT), alkaline phosphatase (ALP) and aspartate transaminase (AST) activities were analysed according to the previously described methods.[34-36] Protein contents in different samples were measured following the method of Lowry et al.[37] Ultra structure of red blood cells of treated rats were prepared following previous report[38] and observed under SEM. Histopathological analysis of vital organs like liver, kidney and intestine was done by fixing tissues in 10% v/v formalin

and dehydrated in a series of ethanol solutions (70, 80, 90, 100% v/v). The tissue samples were processed by using paraffin blocks and histological architecture were examined using hematoxylin and eosin staining. Free radical scavenging enzyme markers superoxide dismutase (SOD) and catalase activity were estimated following the referred protocol.[39,40]

**2.14. Statistics**

All the experimental results were expressed as mean±SE, n = 6. The statistical significance was calculated by using one-way ANOVA following Tukey's post hoc test. $p < 0.05$ was considered to be significant.