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EROSION KINETICS OF PROTEIN COACERVATE ENTRAPPING MOSQUITO LARVICIDE CYCLOHEXIMIDE (CHX) AT VARIOUS pH

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Abstract- A homogenous solution of oppositely charged macromolecules may undergo liquid-liquid phase separation through associative interactions, it can then give rise to a polymer rich dense phase, called coacervates. The dissolution kinetics and the release profile of the inter -molecular aggregates and self-assembled gelatin coacervate material entrapping model larvicide Cycloheximide (CHX), at various pH values were assessed. Sustained release of CHX was observed and read by UV spectrophotometer at 250nm at various time points. The dissolution/ erosion kinetics revealed that the coacervate sustained longer in Phosphate Buffer Saline pH-7.4, than at acidic pH-5 or alkaline pH-8 and doubled distilled water. Efficacy studies were done to assess the susceptibility of the mosquito larvae to the coacervates material and CHX over a period of 100 hours. The probit values of the recorded mortality were plotted against log concentration and LC_{50} and LC_{99} values were determined. The high entrapment efficiency of the coacervate when compared to other systems makes it a good candidate for bio-pharmaceutics, not only human therapeutics but also in pest control management.

Keywords- Coacervates, cycloheximide, dissolution kinetics, release profile, larvicide

Introduction

For application of biopolymers (such as proteins and polysaccharides) in the pharmaceuticals, food and cosmetic industries, their interactions are of major significance in their respective applications. Coacervates are polymer-rich super-concentrated homogenous solutions that remain in equilibrium with the supernatant, where oppositely charged macromolecules, undergo liquid-liquid phase separation. Coacervation has been studied most extensively in aqueous solutions of charged synthetic or biological macromolecules in the last couple of decades [1-5]. Precipitation is a liquid to solid phase transition involving latent heat whereas coacervation is a liquid-liquid phase transition involving entropy gain. The investigation of the basic aspects of coacervation of polyelectrolyte complexes provides a foundation not only for the fundamental understanding of these supramolecular structures but also for their practical uses in protein-related applications and industrial processes [1,2,6].

The repulsive and attractive forces between biopolymers underlie two different phenomena: biopolymer incompatibility and complex formation [7]. The formation of bio-complex arises mainly from electrostatic interactions and is dependent on the ionization degree of the polymers and thus the pH. Bugenberg de Jong [6] describes coacervation as a specific type of complex formation where aqueous colloidal solutions are separated into two liquid phases, a colloidal-rich phase i.e., the coacervate, and a colloidal-poor phase. In simple coacervates, the polymer is salted out by electrolytes, such as sodium sulphate [8] or desolvated by the addition of water miscible non-solvent such as ethanol [8]. Currently, turbidity and light scattering measurements are used to follow the process of coacervation [9,10]. The erosion or chemical degradation release is the characteristic of coacervates - a type of hydrogel system. Although, coacervation has been used in various studies for peptide microencapsulation [11,12] very little attention has been paid to the study of release kinetics of drugs entrapped in these matrices.

The last decade has seen a considerable amount of interest in the gelatin/water based systems that includes: physical and crosslinked gels, micro and nano spheres, simple and complex coacervates etc. Gelatin, a biodegradable polymer is derived from the protein collagen, isolated from animal skin, bones and fish skin, either by alkali [13] or acidic process and thus the gelatin obtained differs in their isoelectric point: 9.0 to 9.4 for alkali and 4.8 to 5.1 for acidic processed gelatin. The net result of this diverse process is a final product-containing component with a wide range of molecular weights. Gelatin is a heterogeneous mixture of single or multi-stranded polypeptides, each has a conformation of extended left-handed proline helix containing between 300-4000 amino acids. Investigation on skin collagen [14] suggest that the chains in the unmodified materials are either very long indeed, or are cyclic.

The gelatin based systems have been exploited for drug delivery applications and for its therapeutic potential [15]. A survey of literature indicates that there has been a lot of work in gelatin - agar coacervates [16-19] but negligible work on application of coacervates entrapping pesticides. Cycloheximide, a known protein synthesis inhibitor, was chosen as a representative pesticide and was entrapped in the coacervate to assess its efficacy, if any in laboratory conditions.

Mosquitoes occupy a valuable niche in the natural world and the genera have been around for more than 100 million years. They provide food for other organisms such as amphibians, fish, birds, spiders, and bats, and control animal populations that spread diseases [20]. World's most dreaded diseases are known to be carried and transmitted by mosquitoes. Mosquitoes are a major vector for human and animal disease, including dengue fever, malaria, filaria-

sis, viral encephalitis and yellow fever. Mosquito control efforts target insects either in the larval stage with compounds called larvicides, or in the adult stage with adulticides. Larvicides are generally more effective than adulticides because the larvae are confined to easily-located bodies of water, while the adults are airborne and mobile and more difficult to find. Spraying and dumping of chemicals have been used in the agricultural area for creating a continued environment of soil nutrients, insecticides, herbicides, and other agro-expedient agents [21].

This study proposes to use simple coacervates of gelatin, prepared by liquid-liquid phase separation by exploiting the electrostatic interactions between oppositely charged segments of this polyampholyte, as a medium for entrapment of a model larvicide drug. More specifically, the work focuses on release and dissolution kinetics of this coacervate system, and to exploit its sustained release for control of mosquito larvae in laboratory conditions using CHX as a representative pesticide.

Material and Methods

Materials

Ethanol was otained from Merck, Germany. Gelatin (Type-B bovine skin extract, 75 bloom, pI = 4.9) was obtained from Sigma Chemicals (USA). Cycloheximide (CHX) was also obtained from Sigma Chemicals (USA). All other chemicals used were brought from Thomas baker, India. All of the chemicals were of analytical grade.

Methods

Turbidity Measurement and Coacervate Formation

The iso-electric pH (pI) of 1% gelatin aqueous solution (prepared by dispersing gelatin at 60°C followed by hydration for 30 to 60 minutes) was measured with pH titration using 0.1M HCl and 0.1M NaOH to vary the solution pH. The turbidity was observed, during titration with ethanol at pH = 4.9 = pI through transmittance measurement by turbidity meter (Brinkmann-910, Brinkmann Instrument, USA) operating at 450nm. At the point of inflection where the turbidity attains its maximum value, the intra-molecular folding and intermolecular aggregate formation occurs that leads to subsequent coacervate droplet formation [10] and drives the system towards coacervation. The high centrifugation of coacervate droplet containing solution at ~10,000 rpm for 30 minutes allows the aggregates to form coacervate phase while the folded gelatin molecules mostly stay in the dispersed supernatant in the form of nano-particles. A typical titration profile is shown in [Fig-1] which also depicts the coacervation pathway. The phenomenology of gelatin coacervation is discussed in our previous work [10].

Dissolution Kinetics

The swelling behavior was studied by suspending the pellet, after centrifugation, in 500ml of phosphate buffered saline (PBS) at different pH and DDW (Double Distilled Water) at room temperature in triplicates.

Drug Entrapment

The Cycloheximide (CHX) was dissolved in chloroform at a concentration of 10mg/ml of organic solvent, and was entrapped in gelatin coacervates at the time of preparation. The % drug entrapment was calculated by:

$$\text{Drug Entrapment} = \frac{\text{Drug}_{\text{total}} - \text{Drug}_{\text{supernatant}}}{\text{Drug}_{\text{total}}} \times 100$$

Release Kinetics

The kinetics of drug release from gelatin coacervates was evaluated using the equilibrium dialysis technique, a method for quantifying drug transport across a dialysis membrane [15,22]. Briefly, 1mm^2 coacervate was suspended in 5ml of PBS and put in the dialysis bag (10 kDa) and then dialyzed at a rotation speed of 50rpm against 500ml of phosphate buffered saline (PBS) at pH 7.4. After a known time period a sample of 500 µl was collected from the solution. The drug concentration in the sample was measured in the spectrophotometer (Bio-Tek Instruments Inc, USA) using KC4 v3.4 software.

Efficacy Studies

Toxicity evaluation was done on larvae of laboratory colonized *Anopheles stephansii,* kept at a standard temperature and humidity. Standard WHO procedure was followed for determining LC_{50} and LC_{99} values. Briefly, tests were conducted in 500 ml glass beakers containing 250ml boiled and filtered water to make it chlorine free. Late III or early IV instar larvae in batches of 40 were grouped and exposed to coacervates, coacervates + CHX, CHX *per se* and solvent as Control group. Four sets of tests were conducted for each group with eight replicates and concurrent controls. Larval food (mixture of powdered liver and brewer's yeast) was provided to all the groups. Beakers were covered with fine muslin cloth netting to secure emerging adults. Observations were taken periodically to record dead/moribund larvae and emerged adults. Tests were conducted under controlled conditions of 27 ± 2°C and Relative Humidity 75 ± 5%. 1ml of solution from the water trough containing larvae was removed periodically and the concentration of the released CHX in the trough evaluated by spectrophotometer at a wavelength of 250nm. This concentration was then plotted against a Standard Curve obtained for the CHX at constant temperature.

The probit values of the recorded mortality were plotted against *log* concentration and LC_{50} and LC_{99} values were determined.

SDS-Page

The 12.5% Polyacrylamide Gel Electrophoresis (PAGE) of the larva protein lysate was run by Laemmli method (24). In brief, the protein lysate was prepared using the FOCUS-Insect Proteome Kit (G Bioscience, USA). In each well 10 ug of protein were loaded and run in stacking gel at 60V and in running gel at 120V. The gel was stained by CBB staining.

Results

Formation of Gelatin Coacervates

We probed the ethanol concentration range from 0-44% (v/v), shown as insets A and B in [Fig-1]. The water molecules will preferentially bind to the alcohol molecules through hydrogen bonding as ethanol is added to water and the resultant binary mixture becomes a marginal solvent for gelatin molecules. Secondly, the dielectric constant decreases significantly facilitating stronger electrostatic interactions between charged segments (both intra and inter) of gelatin molecules [10]. Since, the solution pH was close to the iso-electric point of gelatin, there is no net charge on the polypeptide. Nonetheless, the chemical structure of this biopolymer indicates almost a 1:1 positive and negatively charged patches on this linear random coil molecule [13]. These overlap as the chain contracts due to the decrease in the solute-solvent interaction brought in by the presence of ethanol resulting in bringing charged segments to

each other's vicinity through electrostatic interactions yielding chain collapse. There is a very narrow zone between B and C of [Fig-1] (V = 45 ± 2% v/v) where self-assembly of gelatin aggregates occur and these solutions were centrifuged to extract the coacervate material [9,10].

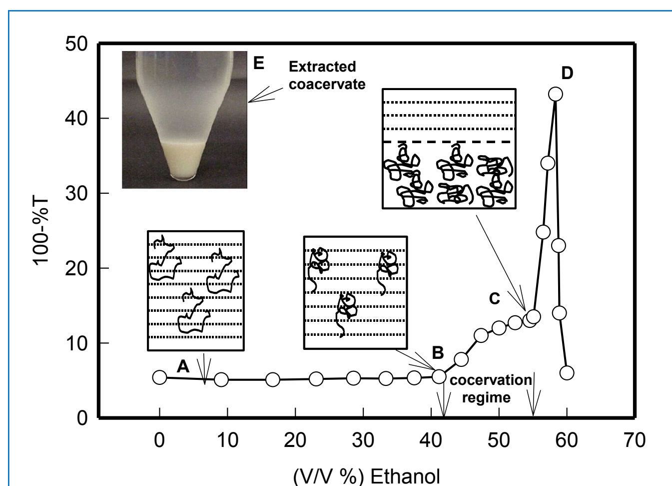

Fig. 1-Titration profile is shown for ethanol/water system for a 1% (w/v) aqueous gelatin solution with I = 0.1M and pH = 5 performed at 25°C. At low ethanol concentration the gelatin molecules have linear random coil conformation (A), as the solvent turns in to a marginal one due to addition of alcohol the chain contracts creating intra and inter molecular chain overlap, only intra molecular folding is shown in inset B. Finally, at a threshold ethanol concentration » 45% (v/v), the liquid-liquid phase transition ensues giving rise coacervation (C). D represents the precipitation point. Top left inset shows coacervate material inside a test tube (inset E).

Dissolution Kinetics

The swelling behavior of coacervates at different pH of PBS and water shows that the coacervates can sustain longer in the PBS pH 7.4 making it an attractive drug carrier [Fig-2]. The swelling behaviour of coacervates in solution is in unison with the swelling behavior of a hydrogel system [25].

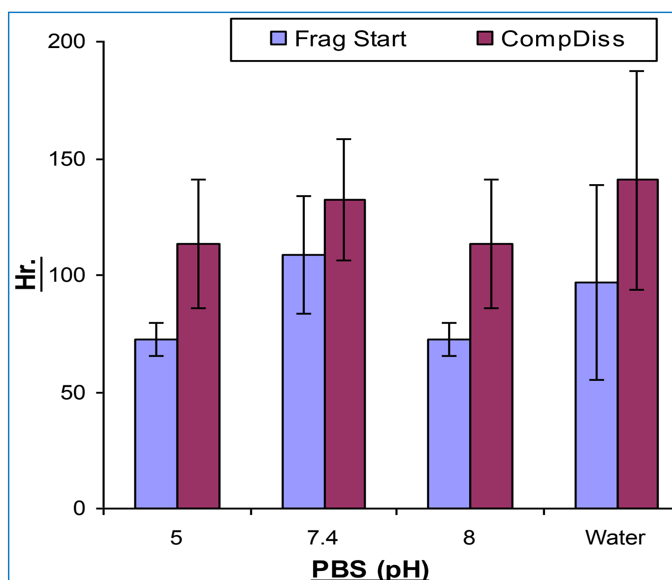

Fig. 2- Dissolution Kinetics of coacervates at different pH and in water

Table 1- Indicates the hours when the dissolution occurs

PBS (pH)	Fragmentation Initiation (Hrs.)	Complete Dissolution (Hrs.)
5	72±6	113±22
7.4	109±20	132±21
8	73±6	113±22
Water (6.8)	97±34	141±39

The entrapment efficiency of the coacervate system is ~70% and is very promising as compared to the nano-scale systems. The coacervate network retains high amount of CHX, with a sustained release of ~20% of drug over 10 days. The drug dissolution kinetics of CHX entrapped coacervates suggest that initially the release was slow for few days and then increased rapidly over the next few days, thereby becoming constant [Fig-3]. The release characteristics, the swelling and dissolution behavior indicate a sustained type of release system, similar to the hydrogel dissolution kinetics having 5 main stages [Fig-3]: (A) Disintegration (B) De-aggregation (C) Release (D) Occultation (E) Occlusion. Details of these are discussed in the given references [24,25].

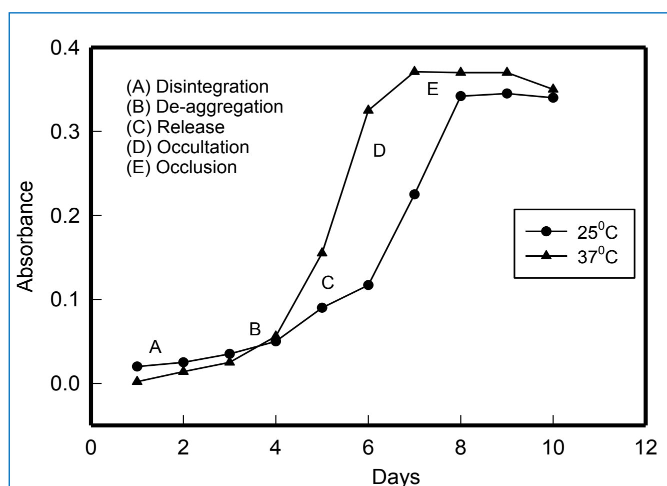

Fig. 3- In-vitro release profile of CHX entrapped coacervate at room temperature and at 37°C. Note the multiphasic release kinetics

Efficacy Studies

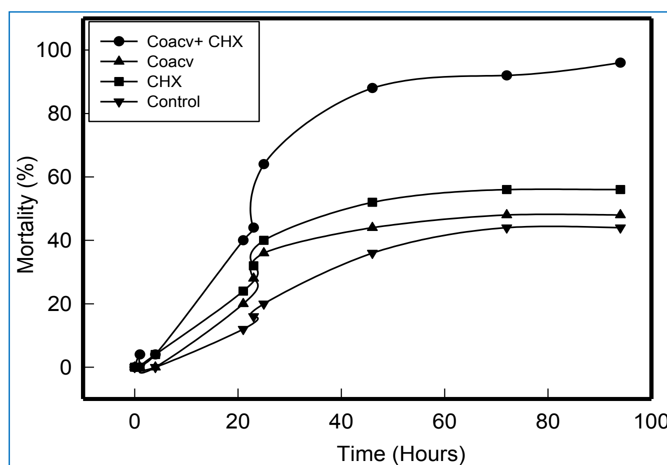

Fig. 4a- Percent mortality as a variable of time. Beakers were periodically checked for mortality of larvae & dead larvae were removed

Baseline data on Coacervate and CHX susceptibility of laboratory bred *Anopheles stephansii* showed 100 percent mortality with 0.212

ppm (n = 40). Mortality with coacervates was 48.5% after 100 hrs. [Fig-4a]. It was observed that the population of *Anopheles stephansii* was fully susceptible to coacervates +CHX after 100 hours (99.04%). But CHX showed 40% mortality at the same time point of 100hr. Probit-logit analysis was done and the calculated LC_{50} = 0.0897ppm, and LC_{99} = 0.2066ppm [Fig-4b].

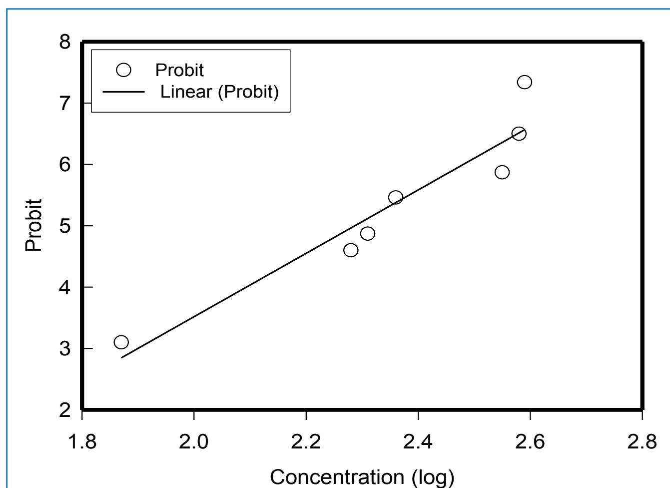

Fig. 4b- Probit *vs* log concentration. Calculated LC_{50} = 179.5 μg/ 500ml (0.0897 ppm), and LC_{99} = 413.31μg/ 500 ml (0.2066 ppm)

SDS-Page

After staining with CBB the 3 bands were visualized in marker lane while in samples lane have two prominent band of molecular weight 94.5 kDa, 58 kDa and one faint band of 78 kDa. The band intensity was comparatively low in the CHX and CHX loaded coacervate lane than the control and coacervate lane [Fig-5].

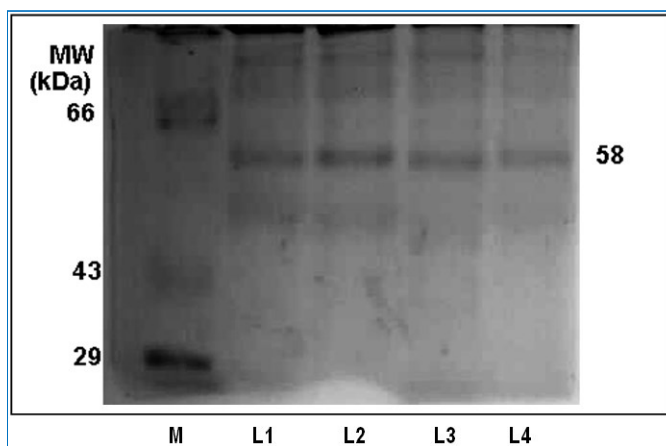

Fig. 5- The SDS-PAGE profile of proteins in the treated and untreated larva. Consistently two prominent band of 94.5 kDa, 58 kDa and one faint band of 78 kDa were present in all the samples (L1-Control; L2-Coacervate; L3-CHX; L4-CHX entrapped Coacervate). In L3 and L4 the band intensity is very less than L1 and L2.

Discussion

The coacervation transition refers to a state of formation of large number of charge neutralized soluble aggregates. Such a situation corresponds to a state where very large number of charged neutralized inter and intra molecular clusters of gelatin molecules are formed in a cooperative manner forming inter molecular clusters. At a threshold ethanol to water ratio the bulk of the gelatin matter is expelled out of the binary solvent mixture along with its hydration water into a separate equilibrium phase called coacervates.

The release kinetics is a complex problem involving concentration of the drug in the formulation, solubility of the drug in base, diffusivity of the drug and the partition coefficient of the drug between the matrix and the release medium. The intra-molecular cross linking of individual coacervates breaks during swellingdue to the water absorption from the surrounding environment and as the water retention of the coacervate/hydrogel increases with time [Table-1], the intermolecular interaction also begins to break causing an erosion of the coacervate/hydrogel network and ultimately releases the entrapped drug [Fig-2]. This disintegration of the network and release profile is largely dependent on the surrounding medium of the coacervate. In an acidic medium (PBS: pH-4.9, pI of the gelatin molecule), The surrounding medium the is allowed to enter the coacervates ie inside the network of gelatin molecule allowing it to swell rapidly. Erosion is hastened in water as compared to PBS at pH-7.4, where, balance between the positive and negative intrinsic charges of the gelatin molecule allows them to retain the water within the network a little longer. But at alkaline pH (pH 8.0) the dissolution seems to be quicker, quite similar to pH-5.0 which can possibly be attributed to the loss in the charge of positively charged groups like imidazole, e-amino containing amino acid - histidine, lysine and hydroxylysine. The pH sensitive swelling and dissolution profile made it a suitable candidate for a sustained release system. The release kinetics and the therapeutic potential of a hydrophobic drug (CHX) entrapped in the hydrophilic matrix of Gelatin has been published earlier [15].

Efficacy studies done to assess the susceptibility of the larvae to the coacervates and CHX, a representative pesticide showed that *Anopheles stephansii* population was fully susceptible to coacervates +CHX after 100 hours (99.04%). But control group and CHX *per se* showed 40% mortality at the same time point (100hr). Probit-logit analysis was done to calculate LC_{50} = 0.0897ppm, and LC_{99} = 0.2066ppm. In an interesting study, Ichwan, et al [26] have encapsulated benzocaine in gelatin-acacia complex coacervates and examined the release kinetics *in vitro*. Microscopic study of the formulations revealed that the coacervate matrix retained its integrity during preparation and storage of the dosage form.

SDS-PAGE conform the action of CHX and CHX loaded coacervate on the larva growth and their death. The expression of 58 kDa molecular weight protein, a key protein as a CHX protein synthesis inhibitor was present in all the samples but in the CHX and CHX entrap coacervate samples it was present in very less amount says to be negligible. So CHX entrap coacervate checks the protein synthesis in larva more prominently than the CHX *per se.*

Once the larval habitats are identified and mapped, our coacervate system can work as a good model for monitoring the mosquito larvae in still waters. Further studies warrant its efficacy in natural breeding areas of the target species for example cement tanks, drums, pits, pools, water fountains and disused wells for *Anopheles stephensii*; and stagnant drains, pits, pools and disused wells for *Culex quinquefaciatus*. The high entrapment efficiency of the coacervates when compared to other systems makes it a good candidate for bio-pharmaceutics not only in human therapeutics but also in pest control management.

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Conflicts of Interest: None declared.

References

[1] Menger F.M. and Sykes B.M. (1998) *Langmuir*, 14(15), 4131-4137.

[2] Brouwer M., Cashon R. and Bonaventura J. (1990) *Biotechnology and bioengineering*, 35(8), 831-836.

[3] Burgess D.J. (1990) *Journal of Colloid and Interface Science*, 140 227.

[4] Cohen Stuart M.A., Besseling N.A.M. and Fokkink R.G. (1998) *Langmuir*, 14, 6846.

[5] Oparin A.P. (1957) *The Origin of Life on the Earth*, 3rd ed., *Academic Press*, New York.

[6] Bungenberg D. and Jong H.G. (1949) *Crystallization-coacervation-flocculation, Colloid Science*, 2, Elsevier, Amsterdam.

[7] Tolstoguzov V. (2003) *Food Hydrocolloids*, 17(1), 1-23.

[8] KHALIL S.A., Nixon J.R. and Carless J.E. (1968) *Journal of Pharmacy and Pharmacology*, 20(3), 215-225.

[9] Kaibara K., Okazaki T., Bohidar H.B. and Dubin P.L. (2000) *Biomacromolecules*, 1(1), 100-107.

[10] Mohanty B. and Bohidar H.B. (2003) *Biomacromolecules*, 4(4), 1080-1086.

[11] Sanders L.M., Kent J.S., McRae G.I., Vickery B.H., Tice T.R. and Lewis D.H. (1984) *Journal of Pharmaceutical Sciences*, 73 (9), 1294-1297.

[12] Sanders L.M., Vitale K.M., McRae G.I. and Mishkey P.B. (1985) *Journal of Controlled Release*, 2, 187-195.

[13] Veis A. (1964) *The Macromolecular Chemistry of Gelatin*, Academic Press, New York.

[14] Bowes J.H. and Moss J.A. (1951) *Nature,* 168, 514-515.

[15] Verma A.K., Sachin K., Saxena A. and Bohidar H.B. (2005) *Current Pharmaceutical Biotechnology*, 6(2), 121-130.

[16] Singh S.S., Siddhanta H.B., Meena R., Prasad K., Bandyopadhyay S. and Bohidar H.B. (2007) *International Journal of Biological Macromolecules*, 41(2), 185-192.

[17] Singh S.S., Aswal V.K. and Bohidar H.B. (2007) *International Journal of Biological Macromolecules*, 41(3), 301-307.

[18] Boral S. and Bohidar H.B. (2010) *The Journal of Physical Chemistry B*, 114(37), 12027-12035.

[19] Lindhoud S., Vries R., Norde W. and Stuart M. (2007) *Biomacromolecules*, 8(7), 2219-2227.

[20] Fealey P.W. (1995) *Killing Mosquitoes without Poison*, The Narragansett Times, Rhode Island.

[21] Edgren D., Leeper H., Nichols K. and Wright (1997) *J. Drug Delivery System*, *New York John Wiley and Sons*, 7, 251.

[22] Allémann E., Leroux J.C., Gurny R. and Doelker E. (1993) *Pharmaceutical Research*, 10(12), 1732-1737.

[23] Laemmli U.K. (1970) *Nature*, 227(5259), 680-685.

[24] Tiwari A., Bindal S. and Bohidar H.B. (2009) *Biomacromolecules*, 10(1), 184-189.

[25] Peppas N.A. (1987) *Hydrogel in Medicine and Pharmacy*, CRC Press, Boca Raton, 3, 95-108.

[26] Ichwan A.M., Karimi M. and Dash A.K. (1999) *Journal of Pharmaceutical Sciences*, 88(8), 763-766.