Introduction

Albumin is an umbrella term for a type of protein which is water soluble. Numerous types of albumin can be found in different living beings and two of the most familiar albumin can be found in egg white and in human blood, known as ovalbumin and serum albumin respectively. They are virtually important to Health and well being for many organisms.

Researches already done shows that the properties of egg albumin and human serum albumin are similar in many properties. The human Serum Albumin and Egg Albumin is similar in their features. Both are utilised in many possible applications like drug loading, spectral properties etc. The natural function of both include activity as carrier for a variety of ligands including fatty acids, amino acids, steroids etc. Albumins are able to transport the hydrophobic ligands throughout the body. It was one of the first proteins to be crystallised and for which the standard purification protocol was developed.

Preparation and application of metal protein conjugates have long been reported. Calcium complex of milk protein casein has been prepared and applied

for developing of a drug delivery system. Most of the reported work involving albumin have used either Human Serum Albumin or Bovine Serum Albumin.

Albumin metal complexes, and their uses in many diagnostic studies such as diagnostic radiology are active research fields .BSA imprinted Polyacrylamide gel beads incorporating functional groups with negative charge are prepared . Such bio imprinted macromolecules are of use in medicine for diagnostics, as biosensors and bio seperation .

Preparation of celecoxib-loaded albumin micro spheres and its application in bio-distribution micro spheres loaded with drug after intravenous administration is reported by Thakkar H and others. Micro spheres were prepared using a natural polymer BSA using emulsification chemical cross-linking method. The prepared micro spheres were characterized for entrapment efficiency, particle size, and in vitro drug release. Surface morphology was studied by scanning electron microscopy. The geometric mean diameter of the micro spheres was found to be 5.46 µm. In vitro release studies indicated that the micro spheres sustained the release of the drug for 6 days .

Synthesis of metallo protein complexes and the study of the influence of metal ions on the structure of carrier proteins reveals that the biological activity of metal is connected with their capacity to bind with metals. Complexes of metals

with natural biopolymers may prove useful in elucidating the role of their structural conformational changes in various pathologies , including malignant degeneration of cells .

Egg albumin is a natural biodegradable non toxic protein. Any preparation from such a natural product could be used for developing drug release system for oral delivery . It will not have any immunochemical response.

Ovalbumin is one of the major protein found in egg white, making up to60-65% of the total protein It is a globular protein and hence soluble in water. Ovalbumin is a carrier protein commonly used as a molecular weight marker for calibrating electrophoresis gel.

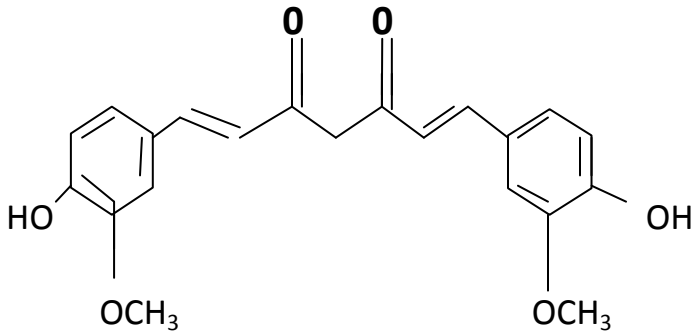
Ovalbumin is a natural protein able to form complexes in various shapes. This protein is biocompatible, biodegradable, nontoxic, and non immunogenic. Due to these features, albumin particles are a good system for drug and antigen delivery. It is a versatile protein carrier for drug delivery, has been shown to be nontoxic, non-immunogenic, biocompatible and biodegradable. Therefore, it is ideal material to fabricate nano particles for drug delivery. Albumin nano particles have gained considerable attention owing to their high binding capacity of various drugs and being well tolerated without any serious side-effects.

CURCUMIN

Curcumin is natural pigment with remarkable pharmacological activity, is a hydrophobic compound and shows lipid solubility. It is a natural product found in rhizome of curucuma longa (turmeric). On going research and clinical trials provide ample evidence that this natural phenolic compound possess diverse pharmacoological potencies. It exhibits anti inflammatory , anti – neoplastic, anti oxidant chemo preventive activities and has been shown to be pharmacologically same even at high doses.

Curcumin 1,7 bis (4-hydroxy-3-methoxy-phenyl)-1,6 heptadienes -3,5-dione. It has also been used to treat disease such as skin wounds and tumors as traditional medicine. Curcumin – protein interactions have been studies and the effect of curcumin on inhibition and activation of protein kinase 'c' is reported.

Structure of curcumin



Curcumin has potential use as a drug. But its use as a drug is limited by the poor solubility in water. If it can be loaded on a suitable carrier to form a suitable compound of appropriate physical properties , the use of curcumin as a drug can be improved.

Aim of the Project

This project to prepare and characterize conjugates of albumin with metal ions like Zinc ,Magnesium, etc. Conjugates involving two drugs curcumin and 5-flouro uracil are also prepared. Drops of solutions are mixed in varying ratios on microscope slides , allowed to dry and the residues examined under a polarized microscope. Anisotropy of crystals are identified with polarized light. Techniques employed for characterization include IR and UV spectrum analysis, particle size analysis and SEM analysis.

MATERIALS AND METHODS

Egg albumin / Ovalbumin

Egg albumin flakes (protein-95%) from NICE chemical (P) Ltd.

Zinc sulphate ($ZnSO_4$)

$ZnSO_4$ AR-99.5%, from NICE chemical (p) Ltd.

Solvents

Petroleum Ether and Toluene are used as solvents. Ordinary soap solution is used as surfactant.

Drug

Curcumin and 5-Fluoro Uracil from Loba are purchased.

Methods

Preparation of saturated egg albumin solution

About 7mg of egg albumin is accurately weighed in an electronic balance. It is then dissolved in minimum amount of water, then successively into 20ml of distilled water in a clean 100 ml beaker. The solution is then centrifuged to get a clear solution.

Preparation of standard $ZnSO_4$

About 28.744g of $ZnSO_4$ is accurately weighed and transferred into a 100ml standard flask. It is then dissolved in minimum amount of water and made up to the mark.

Instruments

IR Spectrometer

An important tool used to gather information about compound's structure, asses its purity and sometimes to identify it. Here infrared radiation is absorbed by molecules and converted into energy of molecular vibration, either stretching or bending. An IR spectrum is a plot of wave number (X-axis) Vs percentage of transmittance (Y-axis).

IR is useful in measuring the degree of polymerisation in polymer manufacture. It has also been successfully utilised in the field of semiconductor microelectronics.

Fourier transforms infrared (FTIR) Spectroscopy

It is a measurement technique for collecting infrared spectra. When the frequency of the infrared light is varied, IR light is guided through an interferometer. After passing through the sample, the measured signal is the interferogram. Performing a mathematical Fourier transform on this signal result in a spectrum identical to that from conventional infrared spectroscopy.

UV Visible Absorption Spectrophotometer

The instrument used in ultra violet spectroscopy is called a UV/VIS spectrophotometer. It measures the intensity of light passing through the sample (I) and compares it to the intensity of light before it passes through the sample (I_o). The ration I/I_o is called the transmittance, and usually expressed as a percentage (%I).

An ultraviolet spectrum is essentially, a graph of light absorbance Vs wavelength in a range of ultraviolet or visible region.

UV/Visible spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds and biological macromolecules.

Polarizing Microscope B A Pol 300

A polarizing microscope is a special microscope that uses Polarized light for investigating the optical properties of specimens. Polarizing microscope is mainly used in geological studies to study geological specimens. For this reason, it is also known as a petrographic microscope. It is used in other scientific fields such as medicine and biology as well.

Polarizing Microscope is also used for the exclusion of thin section. The polarized light microscope is designed to observe and photograph specimen that are visible primarily due to their optically anisotropic character.

Polarizing microscopes are built like regular optical microscope, but are fitted with some extra features. Unlike regular microscopes which use normal light, a polarizing microscope uses polarized light to study specimens. In polarized light, the light waves vibrate in one direction; in normal light, the light waves vibrate in random directions.

It provides all the benefits of bright field microscopy and a wealth of information. It exploits optical properties of anisotropy to give detailed information about the structure and composition of materials. It is based on the phenomena of birefringence. Polarized light cannot be seen by human eyes in normal circumstances. It can, however, be used in polarized light microscopy to highlight features of minerals and other materials. A polarizing microscope uses the birefringent optical properties of anisotropic materials to study them.

Anisotropic materials are solid substances that have several refractive indices; isotropic materials, which include gases and liquids, have only one refractive index. Birefringence or double refraction occurs when a light wave passing through an anisotropic material is split into two rays of differing velocities.

EXPERIMENTS

Preparation of the metal protein complex

Egg albumin flakes from Nice Chemicals are used for the following preparation. The preparation method adopted is direct reaction between a saturated aqueous solution of albumin with aqueous salt solution. Mixture of petroleum ether and toluene is used as an immiscible solvent system to distribute albumin . It is made to react with metal ion by adding 1 M Zinc Sulphate solution. 1 ml petroleum ether saturated with surfactant liquid is taken in a test tube. It is mixed with 2ml toluene. Shaken well 0.3 ml albumin solution is added and again shaken well. 0.3 ml of zinc metal solution is poured into the mixture solution. A thick white precipitate is obtained on vigorous shaking. The whole matter in the test tube is transferred into a centrifuging tube. A few drops of dilute sulphuric acid is added .It is centrifuged for 5 minutes. The supernatant liquid is poured out. The precipitate is washed with distilled water and centrifuged again.

The process is repeated for several times with the same amounts of reagents. The final thick white precipitate is suspended in water. The solid matter is collected together. The complexes obtained are then analysed. The albumin zinc metal

complexes we obtained are washed with several times with water and centrifuged. Then we obtained the pure albumin zinc metal complexes. After the washing processes the albumin zinc metal complexes are separated and made free from the solvents used. The albumin zinc metal complex obtained is then allowed to dry. This dry albumin zinc metal complex is then used for further studies.

The amount of albumin and salt solutions are optimised by trying with variable volumes and viewing under an optical microscope. Calcium complex is also prepared by similar method. The amount of calcium salt solution added is 0.2 ml for 0.3 ml albumin solution.

Particle size analysis is conducted for both the complexes using Malvern Particle size analyser.

Zinc Albumin Conjugate - Bulk preparation.

1 ml Petroleum ether saturated with surfactant liquid is taken in a test tube. It is mixed with 2ml toluene and shaken well. 3ml Zinc metal solution is added from burette to the mixture solution. 3ml ovalbumin solution and 4ml curcumin are added and vigorously shaken . A thick precipitate is obtained. The whole matter in the test tube is transfered into a 250 ml beaker.

The process repeated. The whole substance in the beaker is then stirred countinuously for about 5 minutes using an electric stirrer.

The metal ovalbumin-curcumin complex is formed is then transferred into vaccum evaporator. The temperature of the vaccum evaporator is then set as 80^{o}. The process is carried out for about 30 minutes. After this process we get solid dry metal-ovalbumin-curcumin complex.

Preparation of metal protein conjugate samples on slides

A very small quantity of aqueous solution of ovalbumin is placed on a glass slide using a dropper , allowed to dry and viewed through microscope, the micrographs are taken.

Slides are prepared with varied number of drops of saturated solution of albumin and Zinc Sulphate solution. Samples are compared with respect to the amount and quality of solid samples formed .

The relative quantities of albumin, and zinc salt solution are varied and optimized by trial and error method.

A few slides are also prepared with a drop of surfactant solution, which would facilitate appropriate orientation of molecules

The micrographs was taken with analyser in and analyser out in various stages. Samples are compared under ordinary light and polarized light. Anisotropic substances show specific behavior under polarized light.

Preparation of metal protein drug conjugate -samples on slides

A very small quantity of aqueous solution of ovalbumin is placed on a glass slide using a dropper , a few drops Zinc Sulphate and solution of a drug are placed over

albumun drops and all three are thoroughly mixed. Slides are allowed to dry and viewed through microscope, the micrographs are taken.

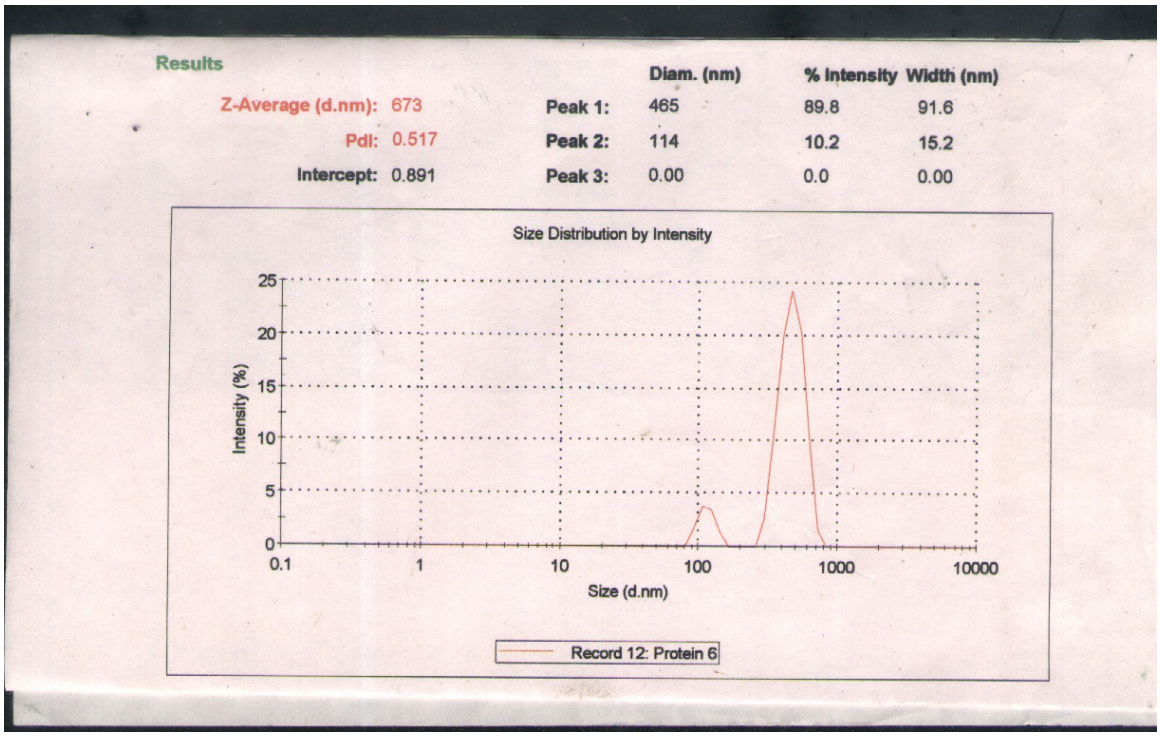
Two drugs are used here ,namely 5- fluoro uracil and curcumin. Curcumin is only very sparingly soluble and so an alcoholic solution is used.

Just as in the case of metal albumin conjugate, variable amounts of albumin, Zinc Sulphate and drug are used on different slides. Samples are compared under the microscope and those slides with characteristic crystalline nature are chosen and micrographs taken.

Result and Discussion

A crude method for preparation of albumin metal complex with zinc and calcium is discussed as above . Figure 1 shows the particle size distribution of the prepared zinc albumin complex. Particles with diameter 465 nm exist with intensity above 90%. Almost single type particles are expected. A very week peak is observed with diameter 114 nm with only 10.2% intensity.

Figure 2 shows the particle size density distribution of calcium albumin complex. Two types of particles are observed in this case. Particle diameter 139 nm is reported with 36.7 % intensity .Another type with diameter 584 nm is observed with 63.3% intensity.



(Figure 1 Particle size Distribution of Zinc Albumin Complex. Two types of particles suggested,with diameter 114 nm and 465 nm.)

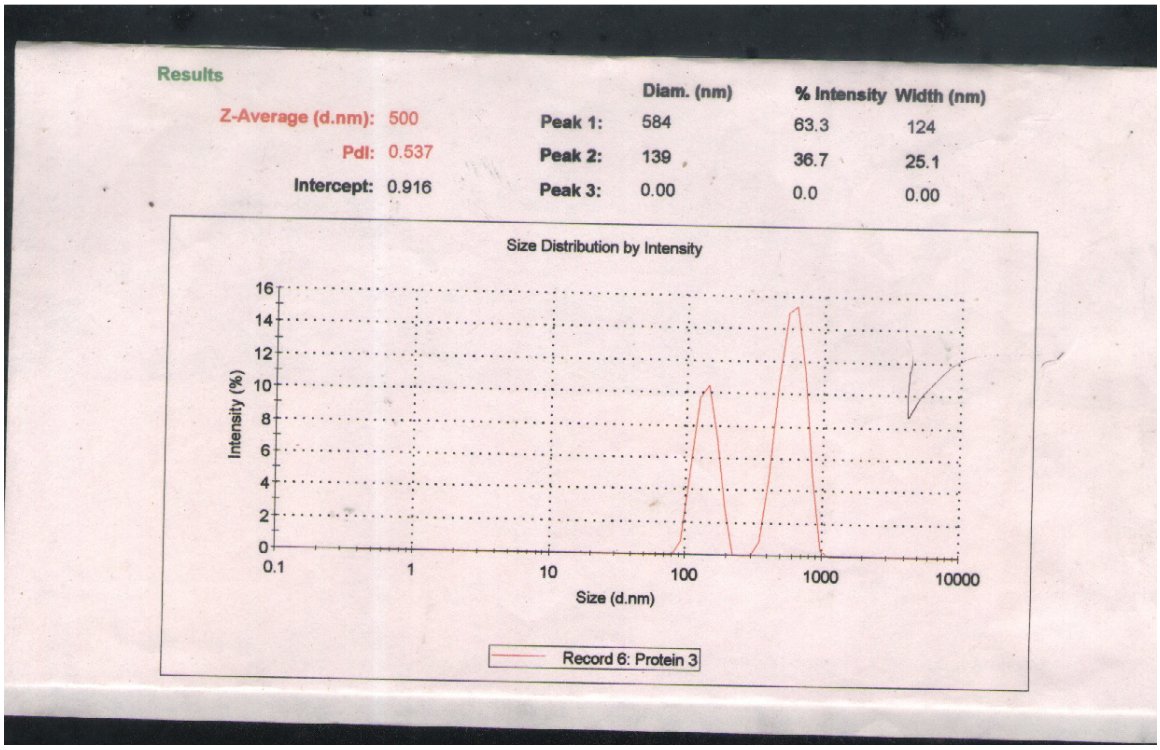


Figure 2 Particle size Distribution of Calcium Albumin complex. Two types of particles with diameters 584 nm and 139 nm observed

Figure3

It is one drop aqueous solution of albumin only .Even the clear saturated solution shows uneven distribution of the solid on the glass plate.

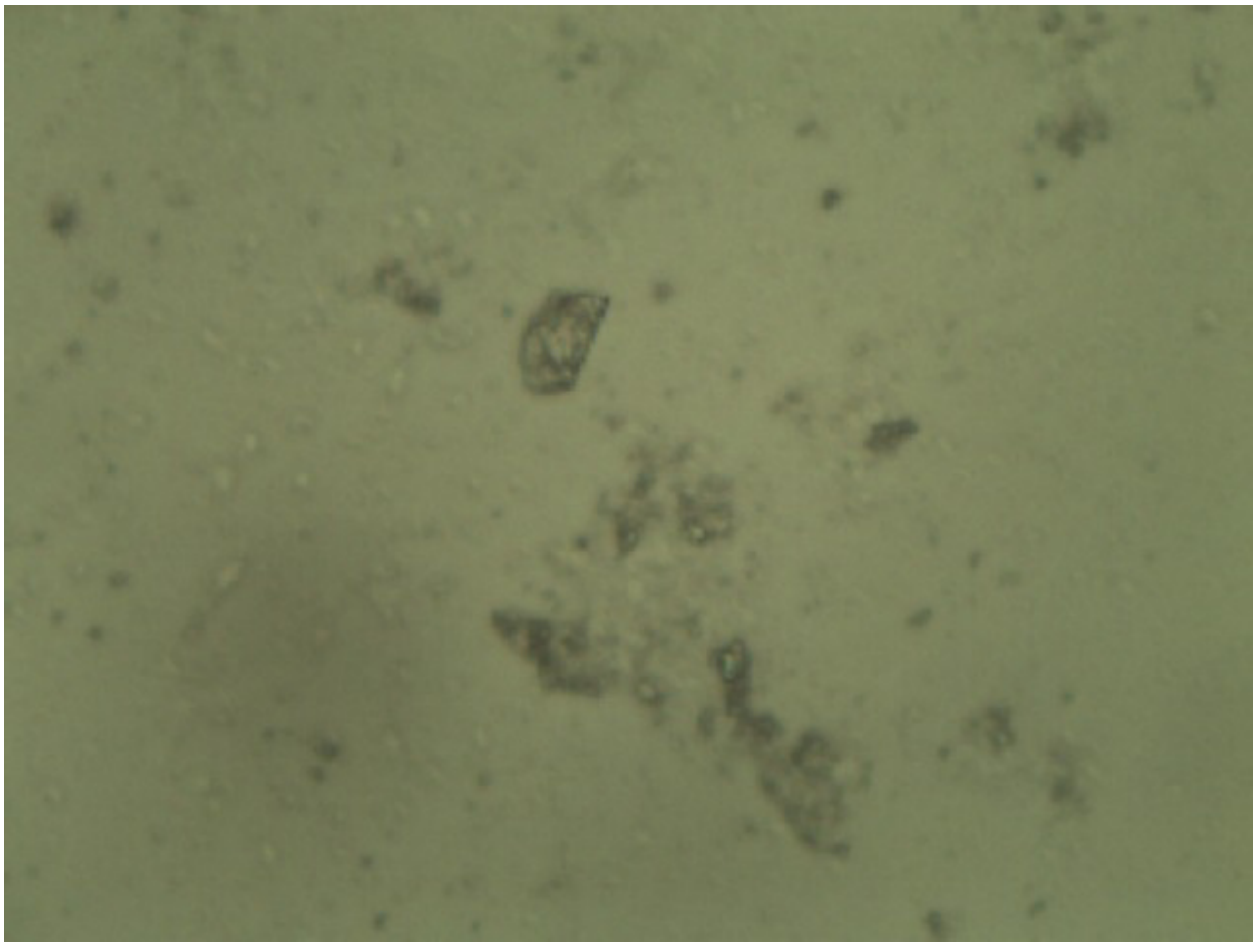


Figure 3 shows dried sample from a few dropd of saturated albumin solution under magnification 10x10. It is not anisotropic.

Figure 4

This shows Albumin with Surfactant. Here Albumin fragments are separated by surfactant .In presence of the surfactant the albumin solid particles are broken in to smaller units , better oriented for combining with other molecules.

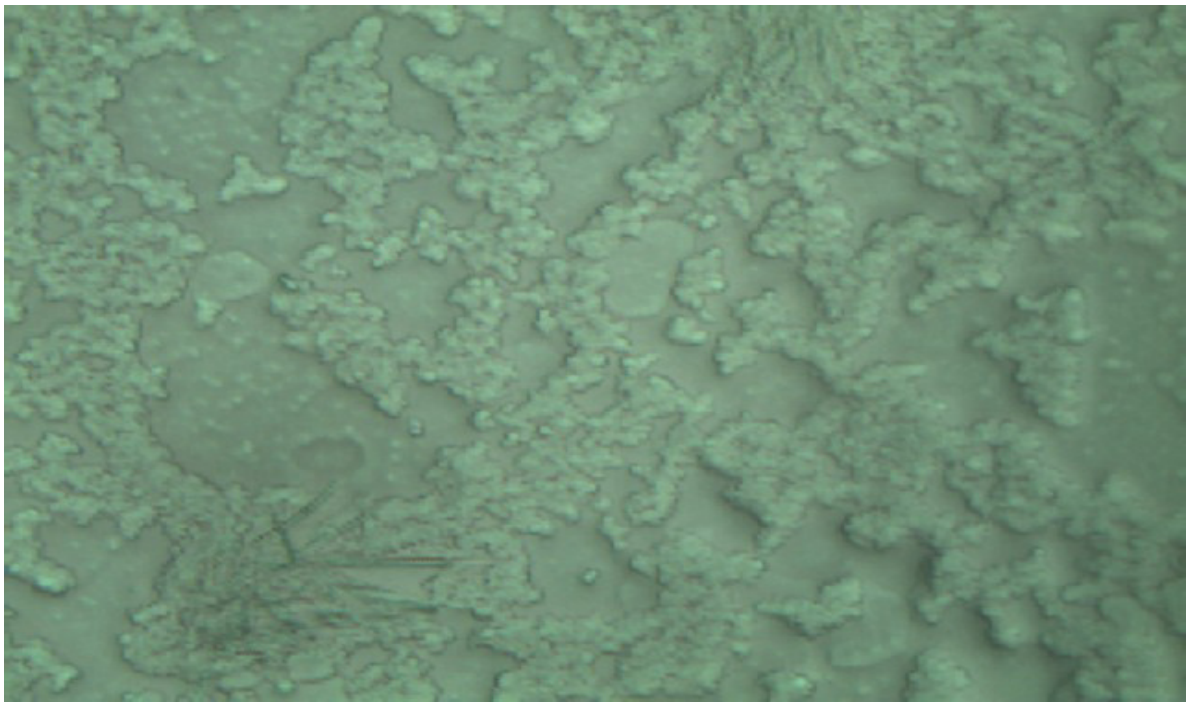


Figure 4 show a drop of albumin solution mixed with a drop of surfactant. It is well distributed over the whole slide. The presence of surfactant facilitates conjugate formation. Magnification 10x10

Figure 5

Careful observation reveals hexagonal shape. Visual image is clearer than the image obtained in the digital camera, Motic. Some crystals are placed above others. Many slides are obtained like this. A few are shown here.Hexagonal crystals of zinc with albumin formed in presence of surfactant.

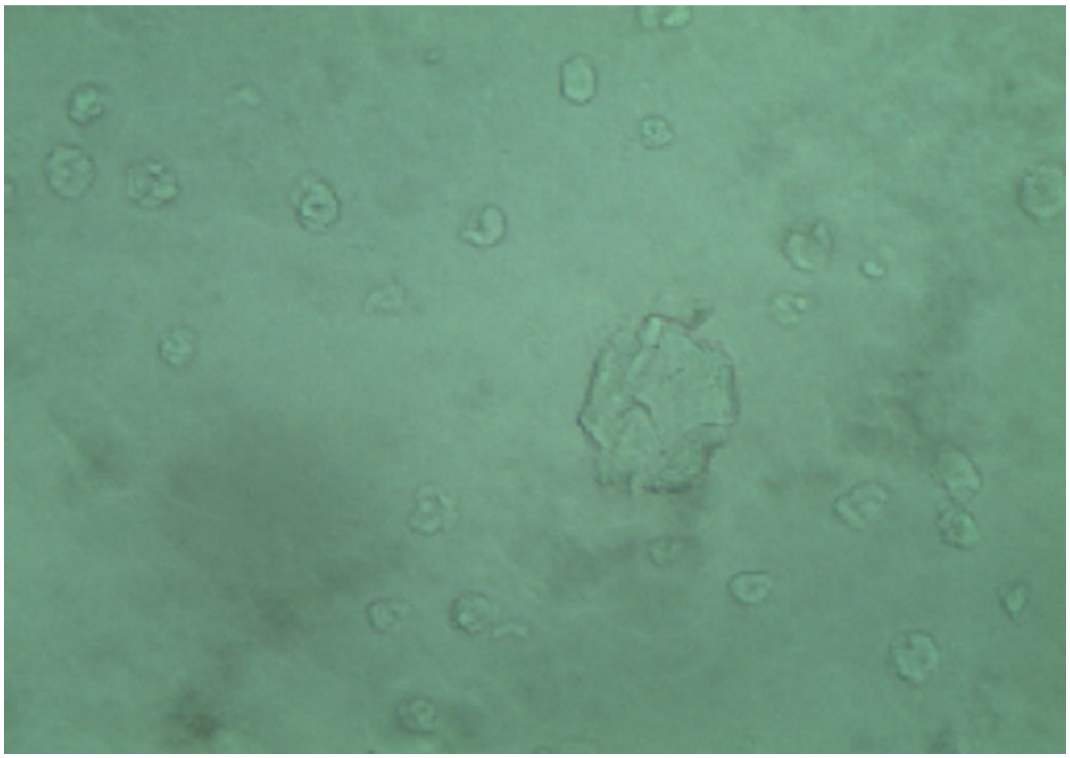


Figure 5 shows sample prepared with one drop albumin and two drops zinc sulphate solution in presence of surfactant under magnification 10x10

Figure 5 and 6 are also taken from slides of albumin solution with surfactant and zinc salt solution, under magnification 10X10 and 40X10

respectively. Formation of zinc albumin complex. We can see the hexagonal crystals formed one above the other.

Figure 6

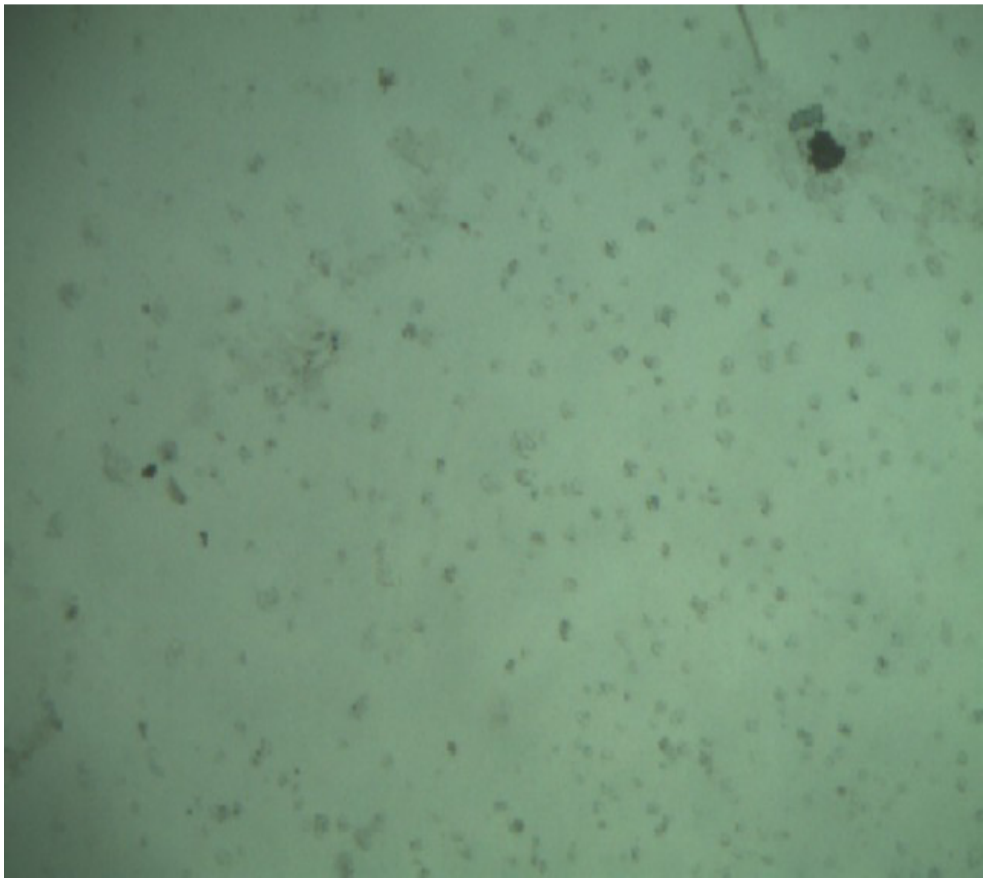


Figure 6 is another slide of zinc albumin Conjugate formed in presence of surfactant

Figures 6 and 7 shows the formation of zinc albumin complex. We can see the hexagonal crystals one above the other.

Figure 7

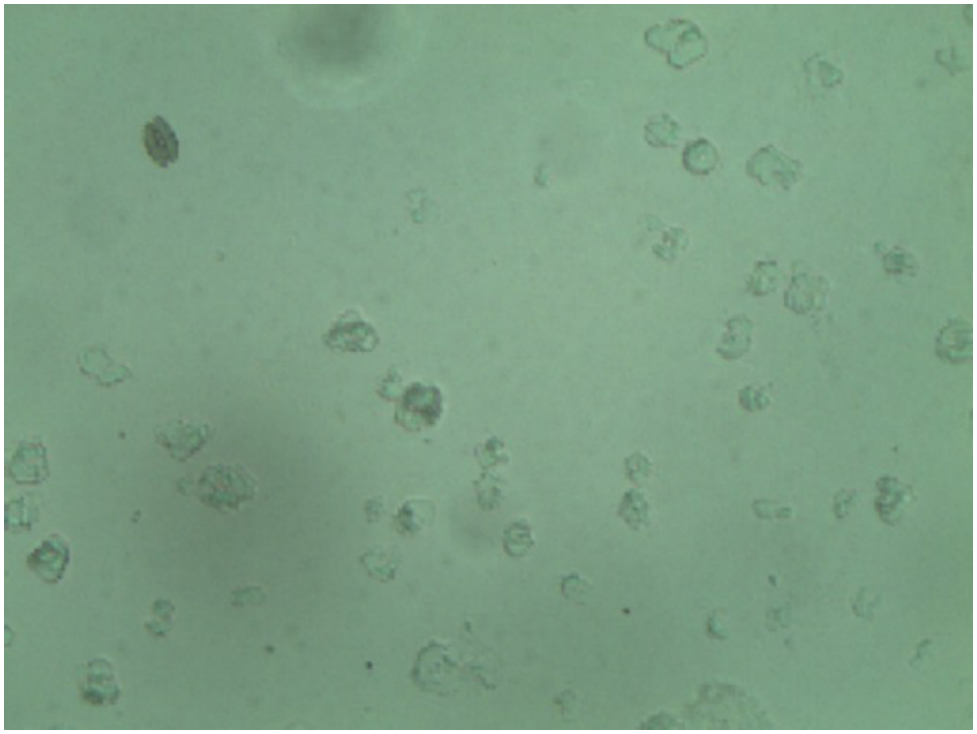


Figure 7 formed by albumin and zinc solutions. The crystals are separated but clustered one above the others. Magnification 10x10.

Micrograghs prepared with equal amount od albumin and Zinc aso gave hexagonal crystals. Figure 8 and 9 are we can see the clear hexagonal crystals under magnification 10X10 and 40x10.

Figure 8

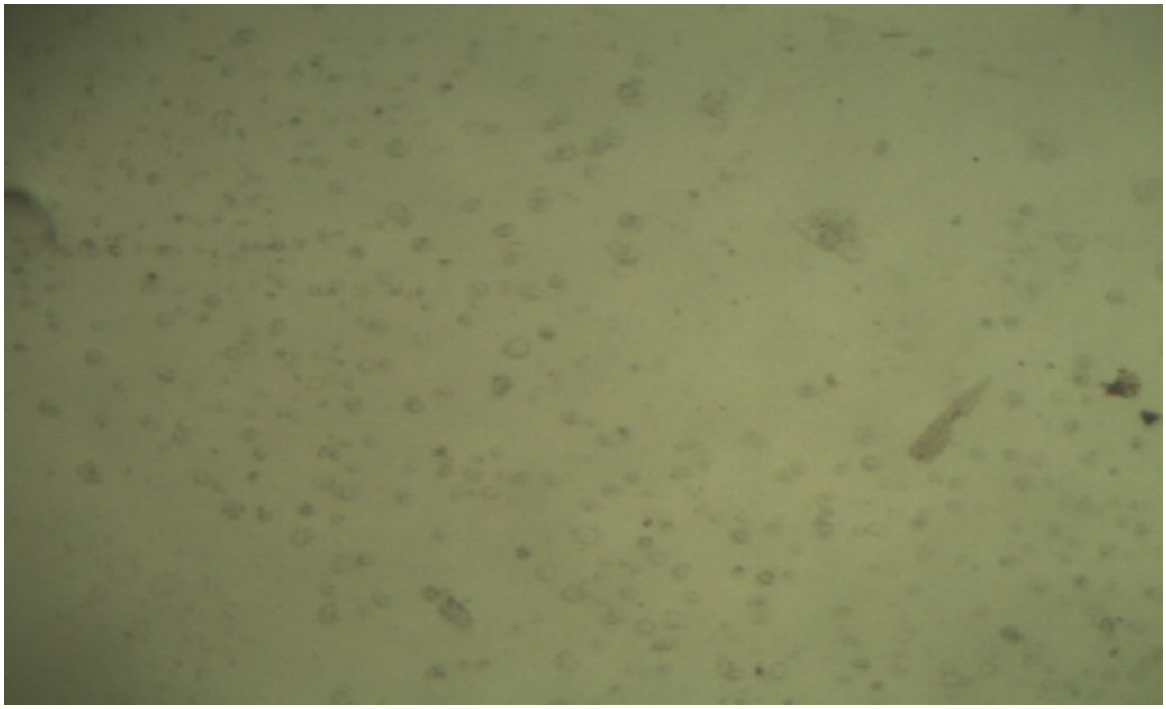


Figure 8 Hexagonal crystals from one drop albumin and one drop zinc .Magnification 10x10

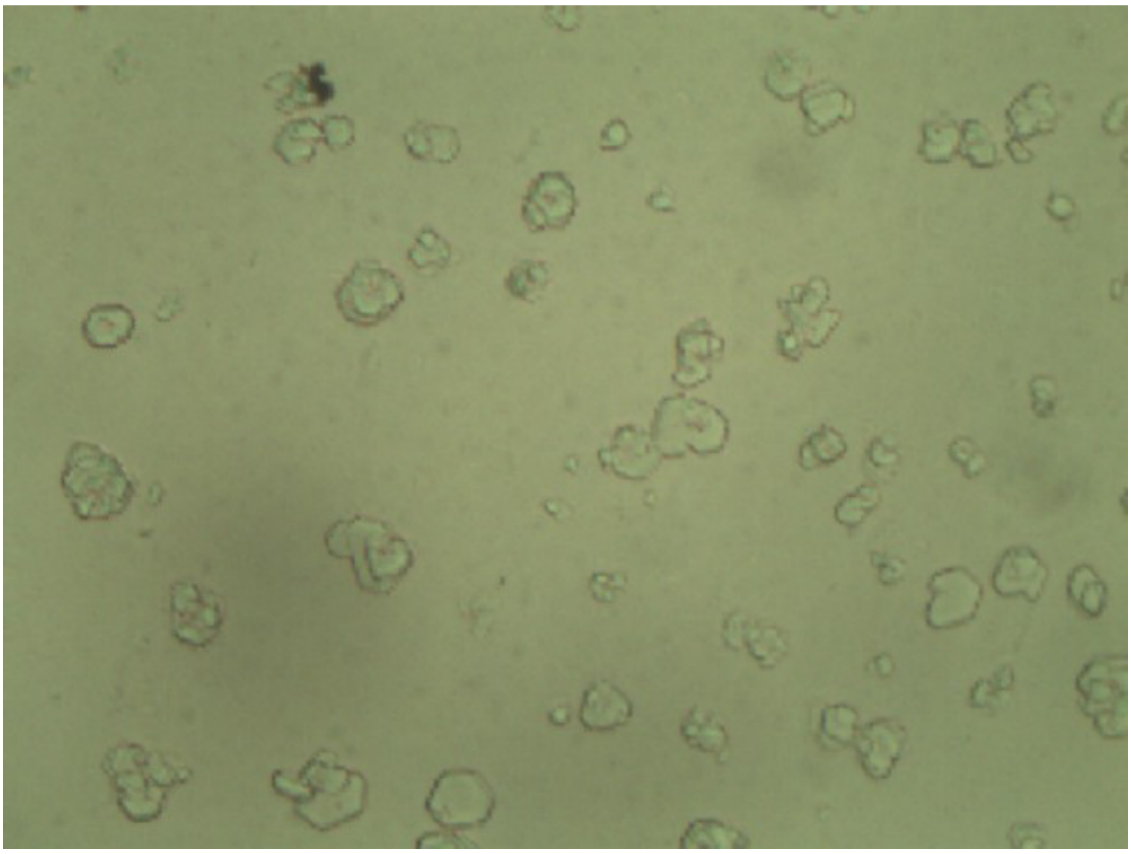
. 

Figure 9 shows a slide prepared with equal no of drops of albumin and Zinc under magnification 40x10

In this combination the ratio of zinc and albumin are equal.

Micrograph 8 shows albumin zinc complex prepared without adding surfactant.

We can see unreacted albumin , but hexagonal crystals can also be seen in between lumps of unreacted albumin.

Figure 10

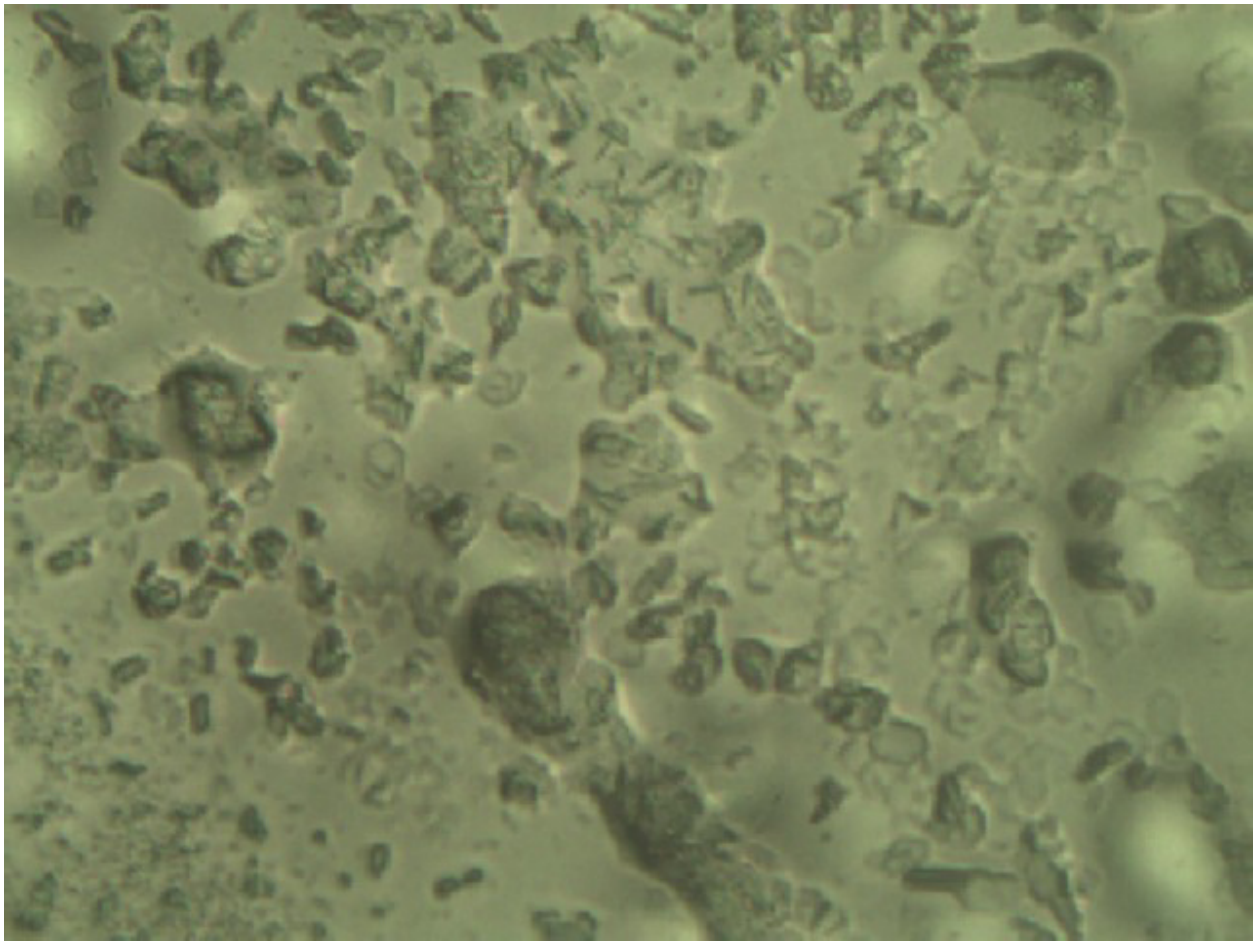


Figure 10 Slide with one drop albumin and 2 drop zinc without surfactant.Magnification 10x10

Here can see the formation of zinc albumin complex even though surfactant is not used. Hexagonal crystals are formed.

Conjugates with Drug

Micrographs 9, 10 and 11 shows mixtures of albumin solution, zinc salt solution and curcumin solution in presence of surfactant. A few needle like crystals are observed when curcumin solution is added.

Figure11

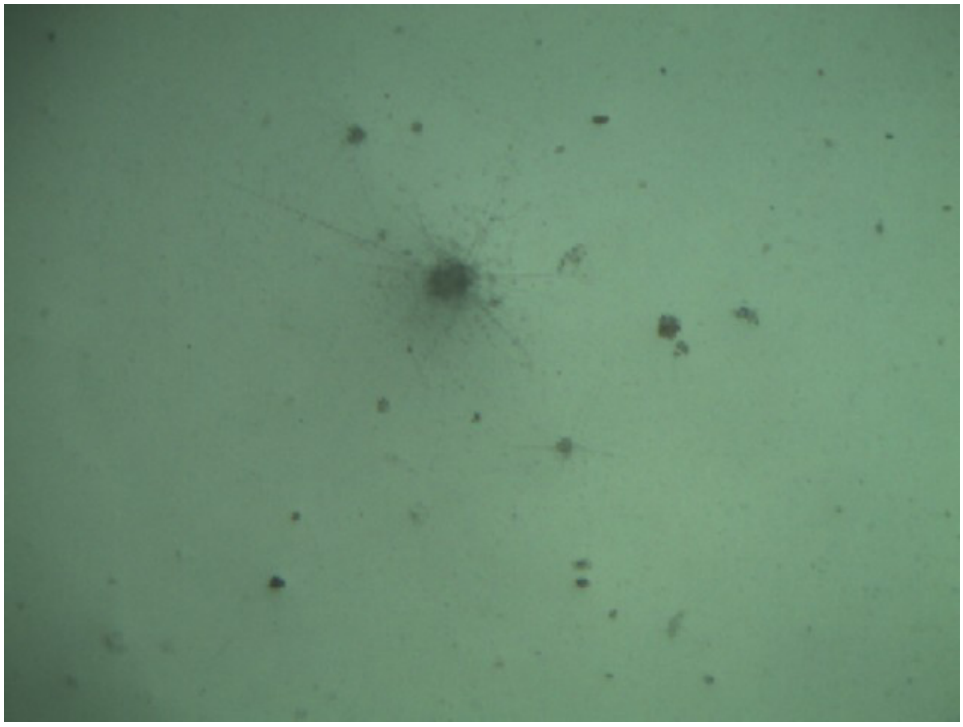


Figure 11 Curcumin conjugate .very few crystals are formed .viewed under 10x10

Figure 12

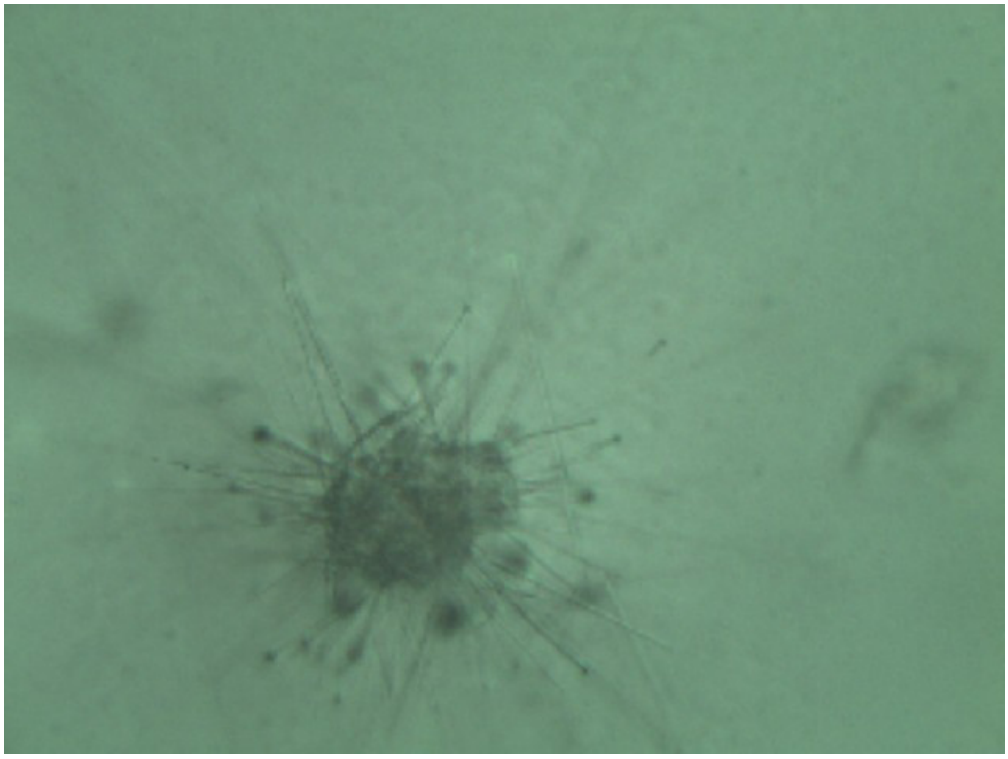


Figure 12 Curcumin conjugate .very few crystals are formed .viewed under 10x10

Figure13

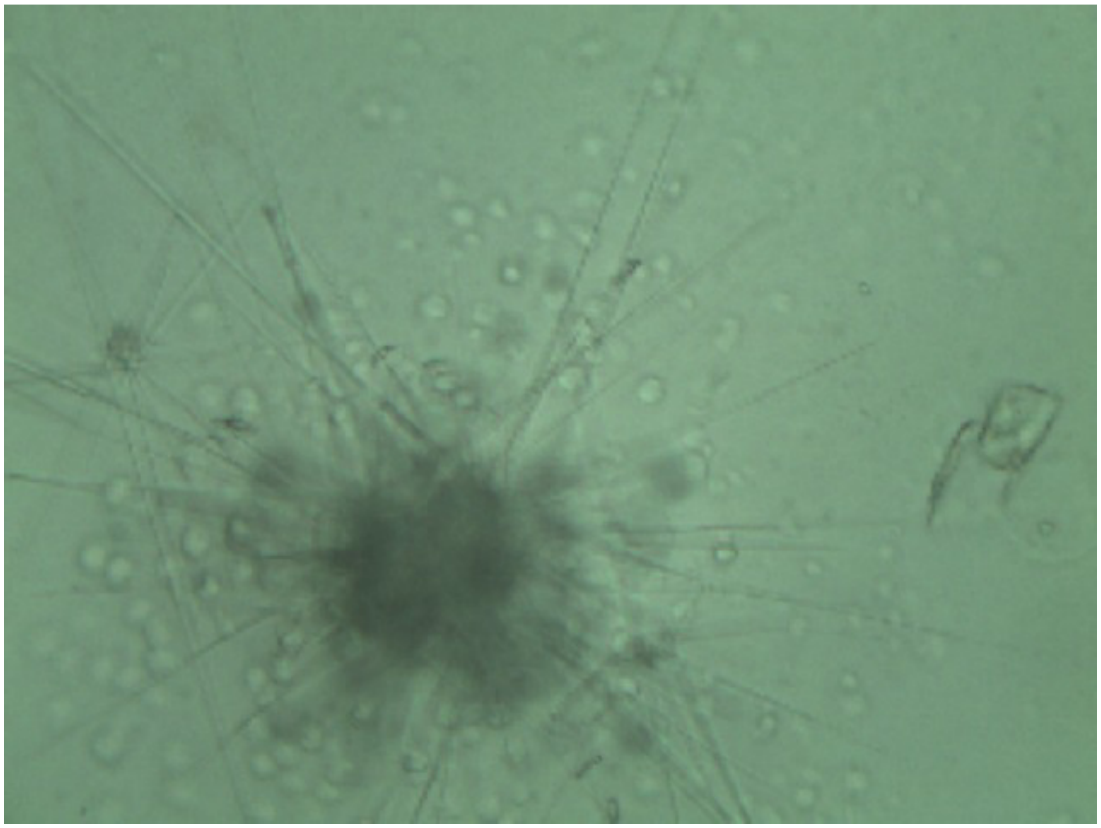
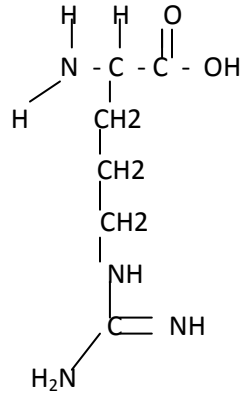


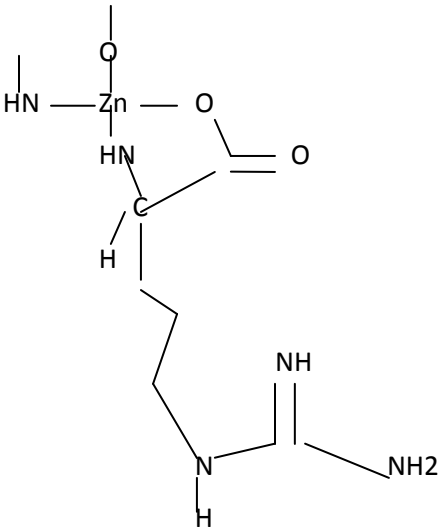
Figure 13Curcumin conjugate .very few crystals are formed .viewed under 40x10

This figure shows the formation of zinc-albumin –curucumin complex. Here we can see sharp needle like crystal near curcumin. This may be due to the complex formation of zinc albumin complex with curcumine. Here we can see formation of zinc- albumin complex. Hexagonal crystals are due to the formation of Zn-albumin complex. These crystals placed one above the other. The possibility is that the long albumin chain many have broken, and segments bind with metal ion.

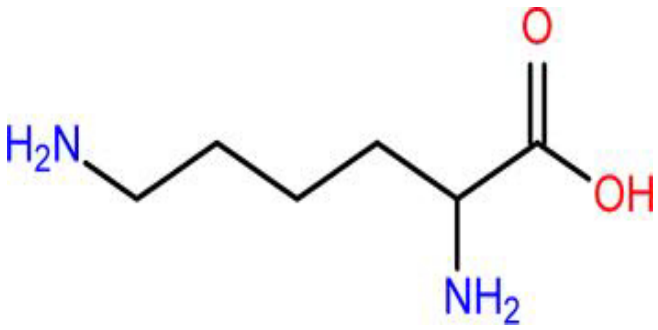
For example, in the structure of ovalbumin the end part consists of Arginine amino acid having the following structure.



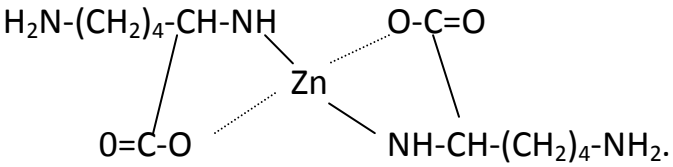
This amino acid may complex with zinc metal as follows,



Another amino acid that present in ovalbumin is Lysin having the following structure.



This amino acid may complex with metal as follows



Curcumin may complex with end Tryptophan, an amino acid present in ovalbumin in the following way.

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SE M analysis

b) ANALYSIS USING SCANNING ELECTRON MICROSCOPE

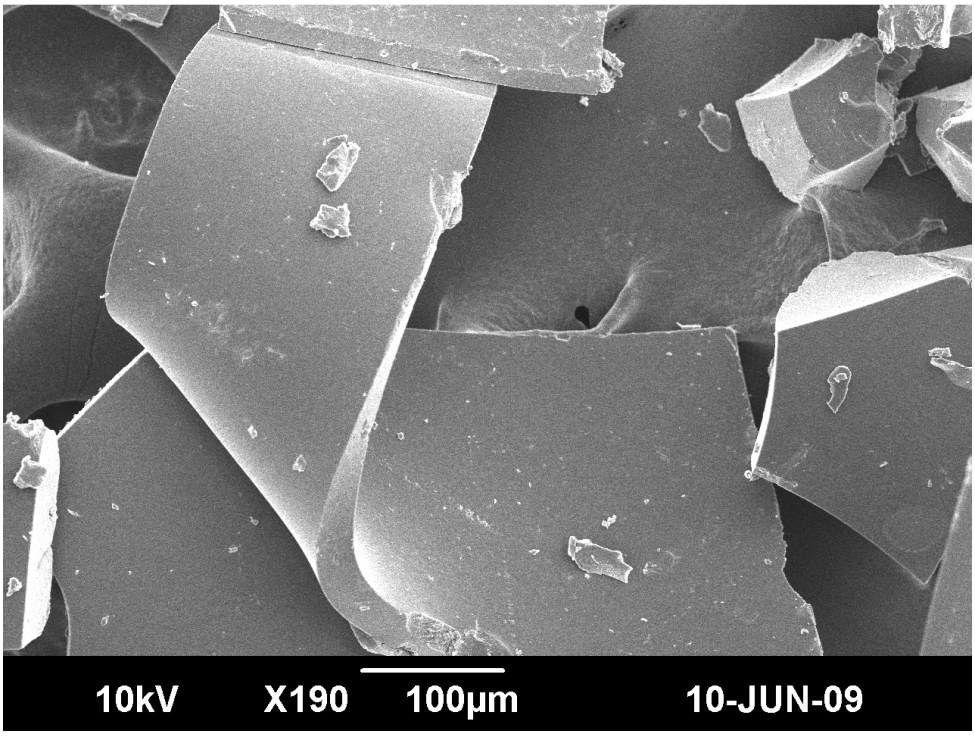


Figure 14 SEM of Albumin .A few drops of Albumin solution placed on glass slide is dried and the sample collected for SEM analysis.

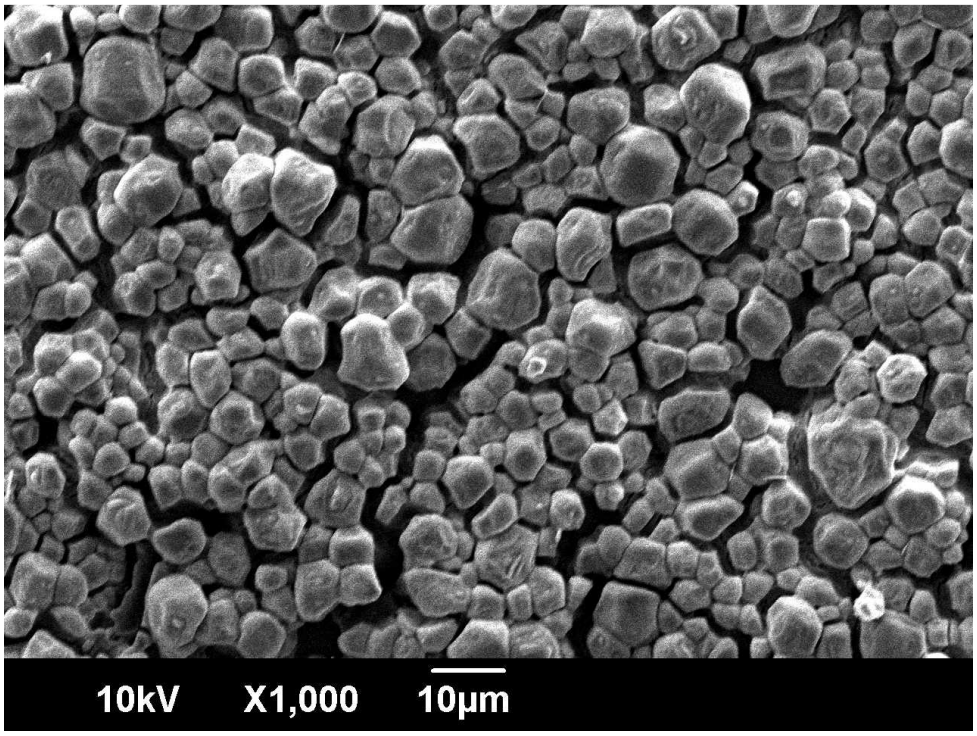


Figure 15 SEM of Zinc Albumin conjugate from slide containing one drop albumin and two drop Zinc Sulphate solution.

The SEM image in Figure 15 clearly suggests formation of crystals of micrometer size range . This is in agreement with the conclusions from observation with Polarised microscope.

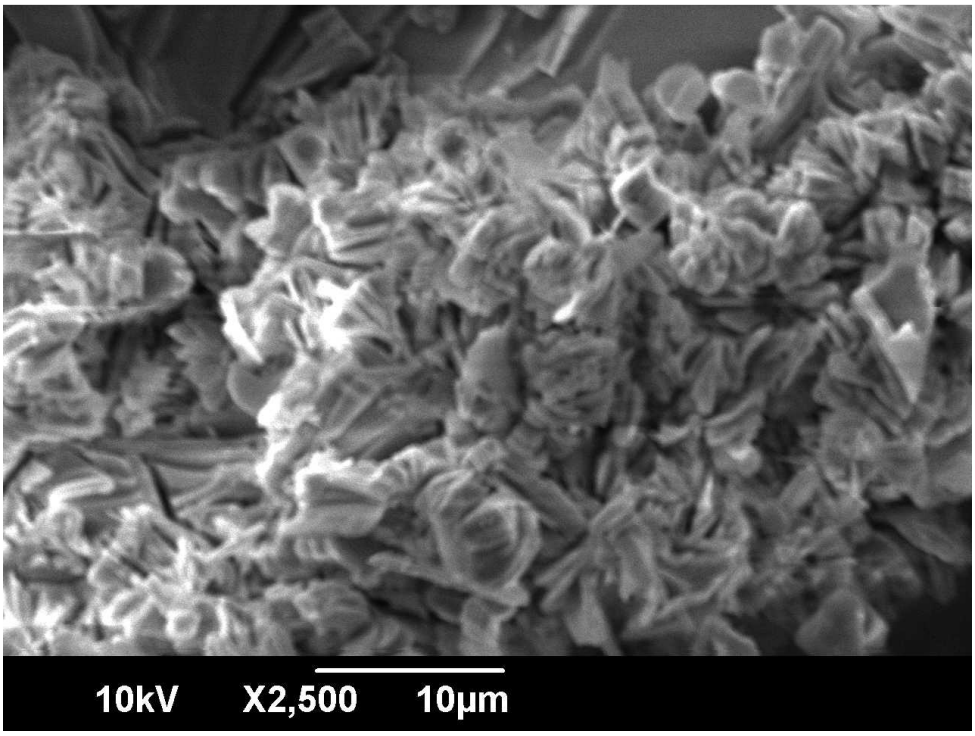


Figure (4)

Figure 14 is SEM of drug conjugate collected from slide containing two drop of albumin solution in alcohol, two drop of zinc in alcohol and one drop of drug 5-fluoro uracil in alcohol

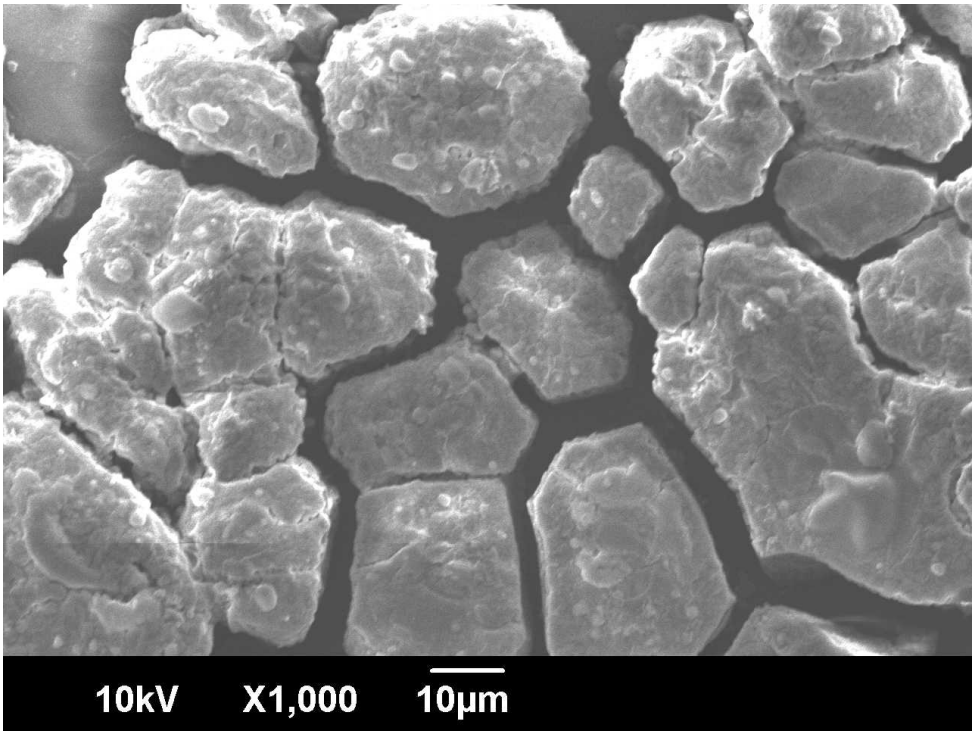


Figure 16 SEM of curcumin conjugate from slide with two drop of zinc solution , two drop of albumin solution in water ,in water and one drop of drug solution.

Spectral analysis

Ovalbumin -IR Spectrum

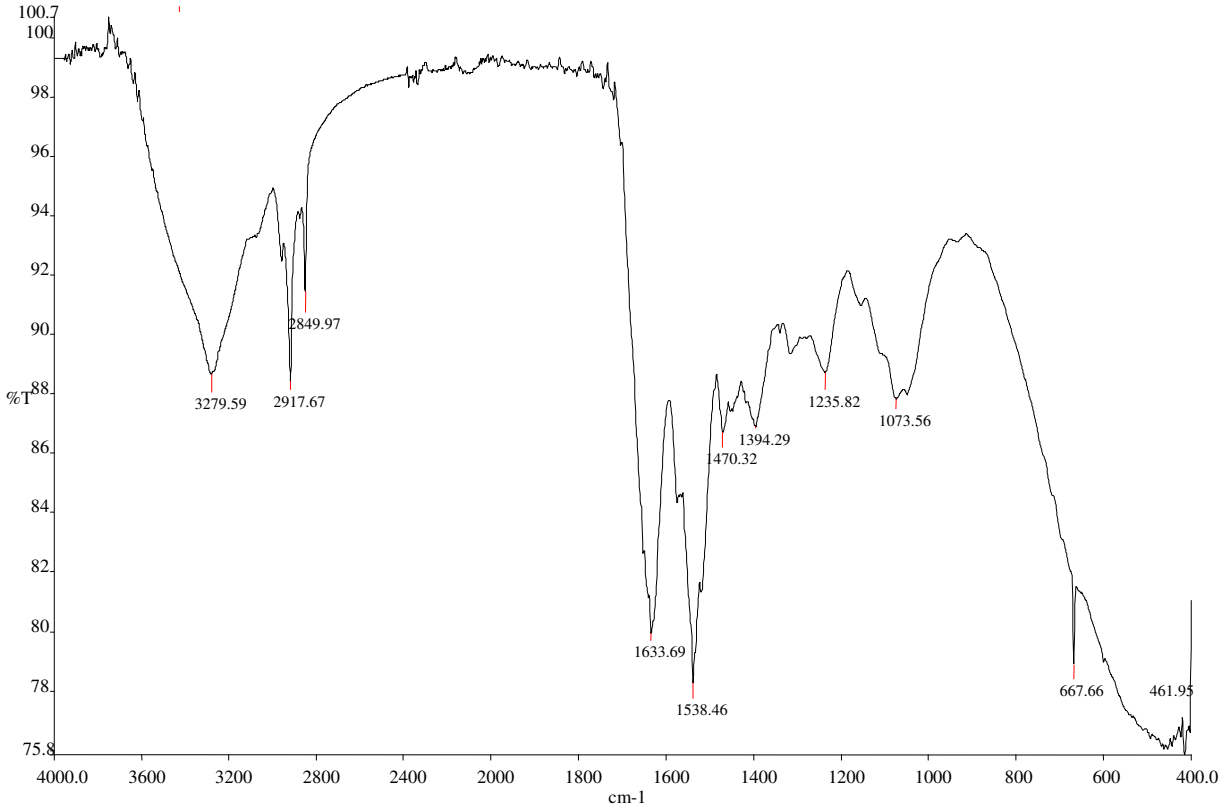


Figure 17 shows the I R Spectrum of Albumin .Sample is prepared from drying aqueous solution.

Zinc Ovalbumin complex-IR Spectrum

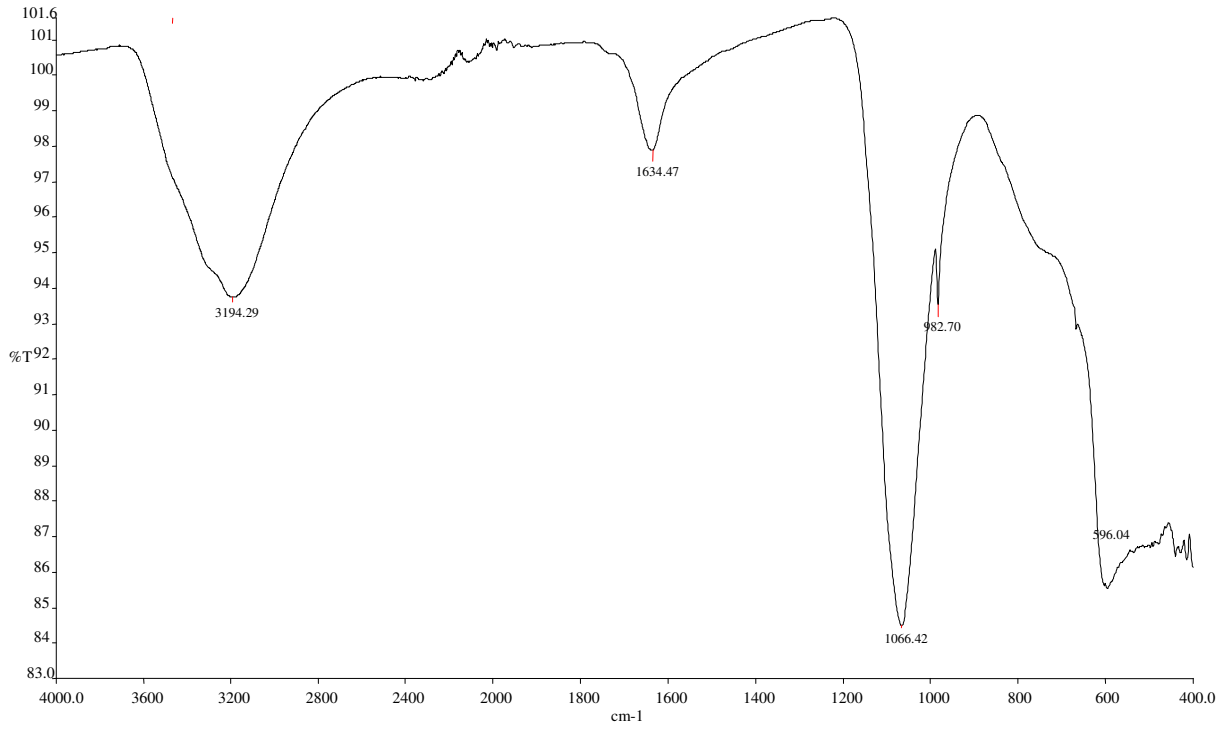


Figure 18 I R Spectrum of Zinc Albumin Conjugate

IR spectra of ovalbumin (Fig 17) and the Zn-Ovalbumin complex (Fig 18) are compared.

Figure 17		Figure 18	
Peaks in cm^{-1}	**Reason**	**Peaks in cm^{-1}**	**Reason**
3279	Due to –NH stretching	3194	Due to –NH stretching
2917,2850	Due to –CH stretching		
1633	Due to C=O stretching	1634	Due to C=O stretching
1538	Due to –NH bending		
1074	Due to –C-O stretching	1066	Due to –C-O stretching
1317,1237	Due to –C-N stretching		

Many of the specific representation in the Ovalbumin spectrum are missing in the Zn-Ovalbumin complex. -NH stretching at 3279 cm^{-1} in Ovalbumin broaded and shifted to 3194 cm^{-1} in the complex. –CH stretching at 2917cm^{-1}, -NH bending at 1538cm^{-1} and –CN stretching at 1317cm^{-1} and 1237cm^{-1} are absent in the IR spectrum of ovalbumin Zinc complex. C-O stretching at 1074 cm^{-1} shifted to 1066cm^{-1} in the complex. The IR spectrum of complex also consists of a specific peak at 982 cm^{-1}. These changes are expected to be due to the binding of Zinc with certain functional group such as –NH and C-O.

UV/V Spectral data analysis

Figure 19

UV Spectrum of Ovalbumin

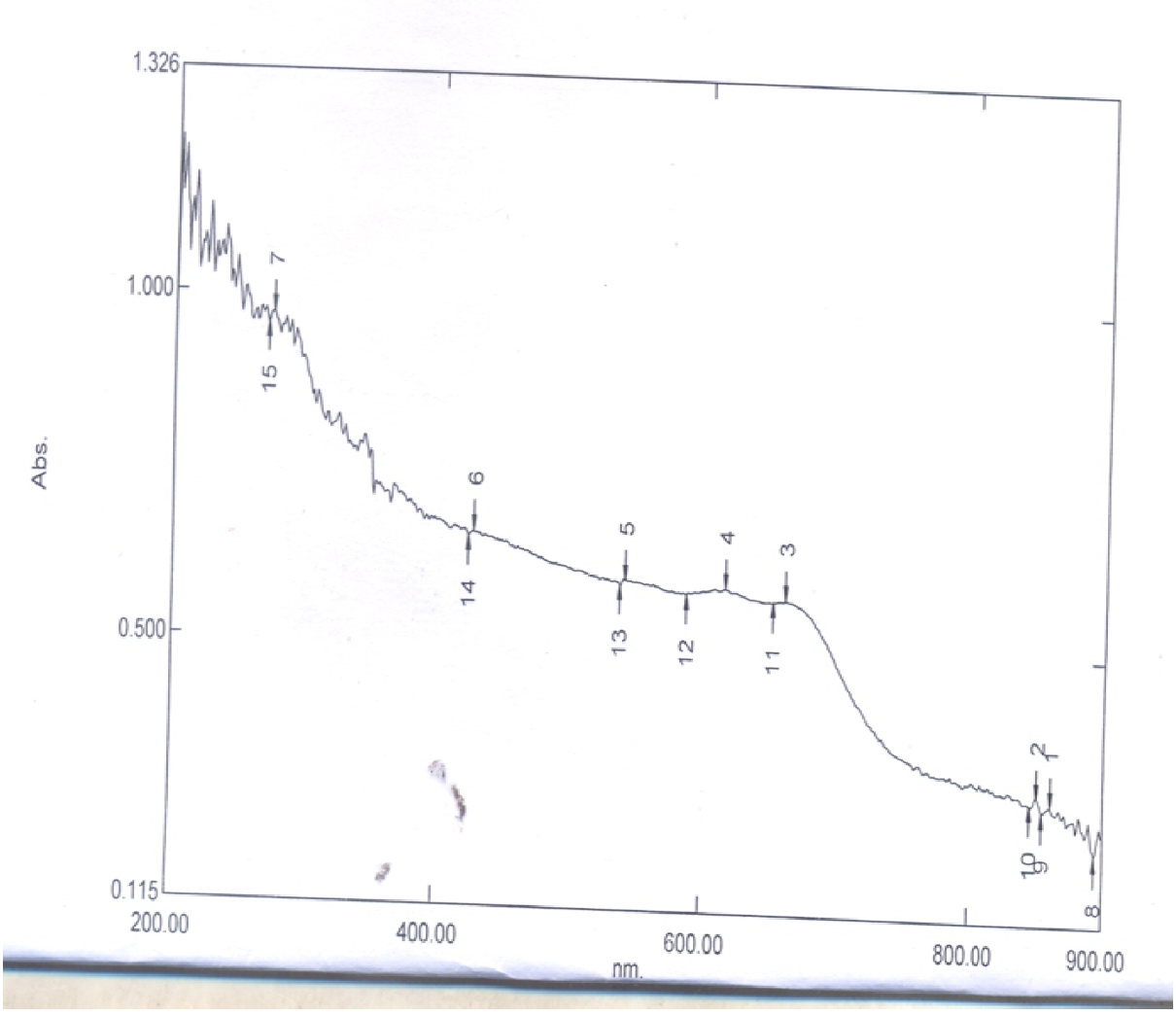


Figure 19 U V Visible Spectrum of Albumin

In the UV spectra of ovalbumin (Fig 19) gradual increase in peak height is observed in 400-800 region. These higher wave length peaks corresponds to lower energy n-$\prod^*$ transition. The continuous peak in 300-200 range corresponds to high energy $\prod$-$\prod^*$ transition. This shows ovalbumin has conjugation in both visible and UV region.

Figure 20

UV Spectrum of Zn-Ovalbumin Complex

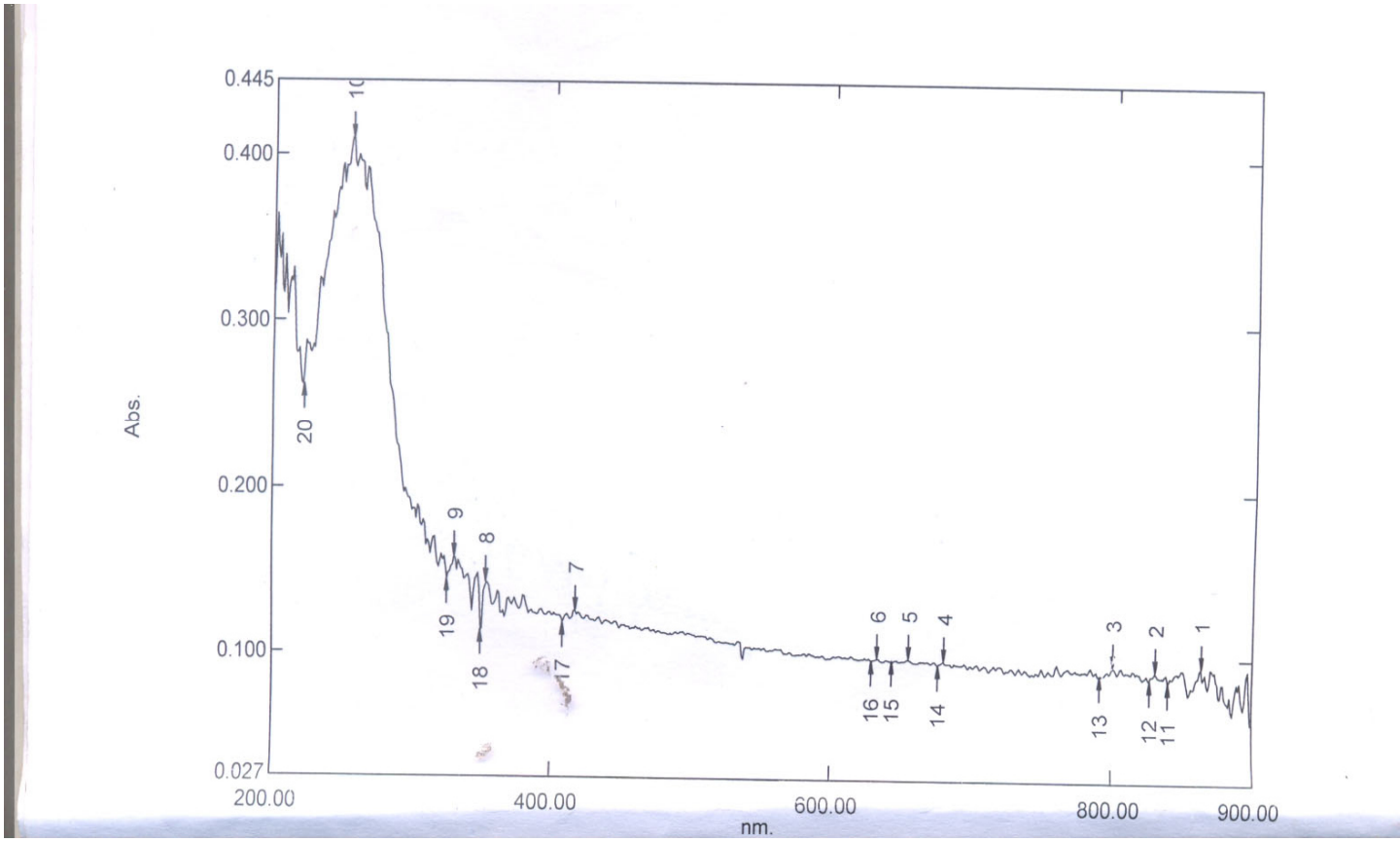


Figure 20 UV-Visible Spectrum of Zinc Albumin Conjugate

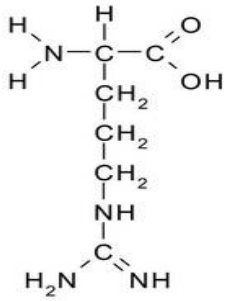
The spectrum of Zn- Ovalbumin complex (Fig 20) shows sharp peaks at 290 nm due to Ligand to metal charge transfer and very small continuous peak in high wave length region.

The spectral data shows that in complex, conjugation in visible region (400-800) is reduced or almost destroyed. This explains the white colour of complex.

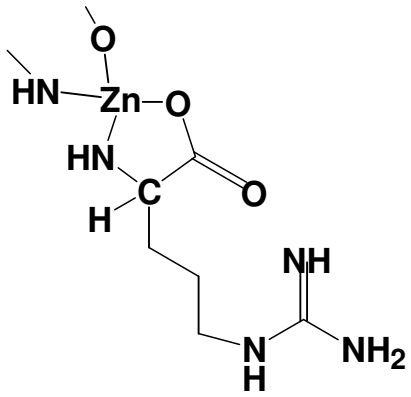
Even though strong peak is formed in complex, the absorbance is decrease from 1.25 to 0.35 and strong peak comes in 0.41 absorbance range. This shows that only small energy is needed for excitation.

Thus a UV/V spectrum also shows the possibility of complex formation between amino acid and metal ion.

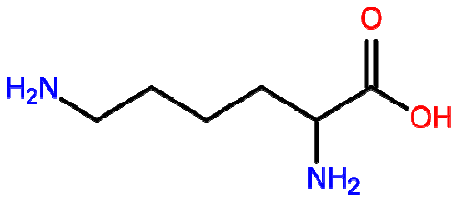
The variation in both IR and UV spectrum on complex formation indicates that metal- albumin complex bond is formed. A possibility is that the long albumin chain may have broken, and segments bind with metal ion. For example, in the structure of ovalbumin the end part consists of Arginine amino acid having the following structure



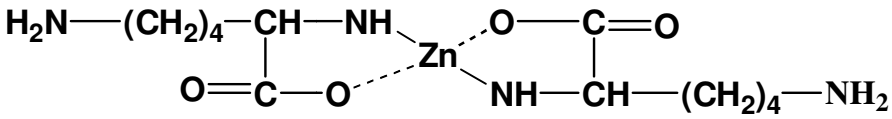
This amino acid may complex with Zinc metal as follows,



Another amino acid that present in ovalbumin is Lysine having the following structure,



This amino acid may complex with metal as follows,



Anisotropy

Analysis using polarizing microscope

Micrographs of ovalbumin under polarized lightwith magnification 10x10

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Figure 20 Albumin Crystal under Polarised Microscope

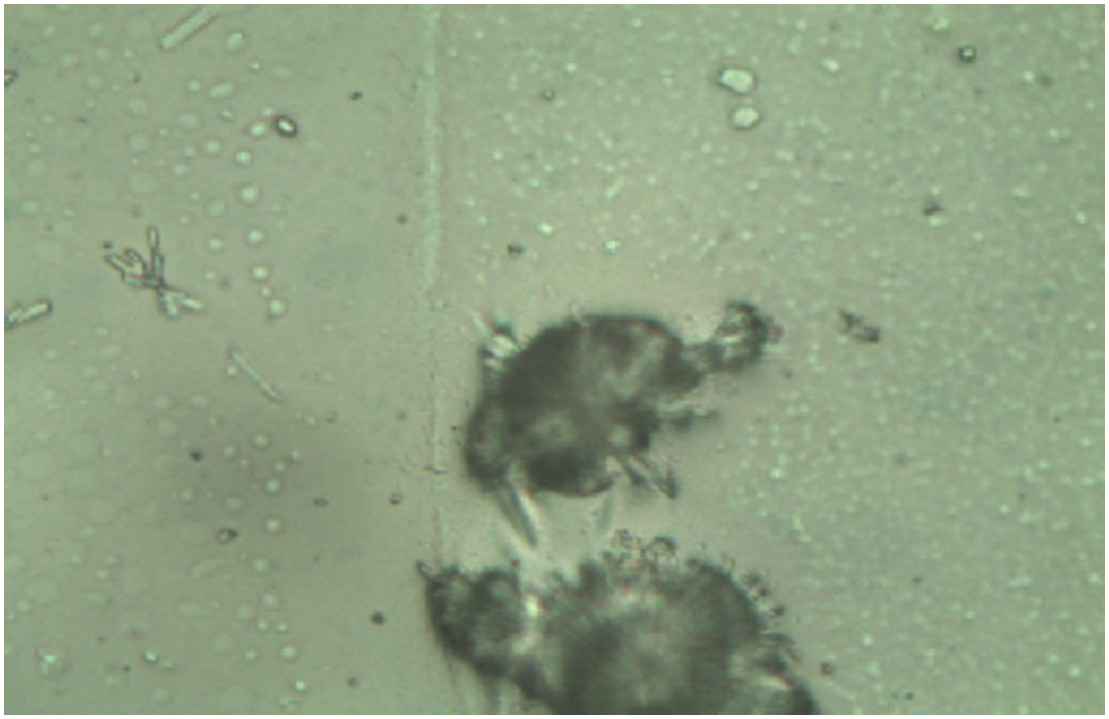

Figure 21 Micrographs of ovalbumin under polarized lightwith magnification 40x10

The micrographic studies of Ovalbumin and its Zinc complex reveals that the crystal structure of ovalbumin is needle like and Ovalbumin-Zinc complex shows a hexagonal structure. This indicates the possibility of hexagonal structure of the Zn-Ovalbumin complex.

Polarising microscope based on the phenomenon of birefringence mainly helps to identify whether the substance is isotropic or anisotropic. In a single crystal, the physical and mechanical properties often differ with orientation. It can be seen from looking at our models of crystalline structure that atoms should be able to slip over one another or distort in relation to one another easier in some directions than others. When the properties of a material vary with different crystallographic orientations, the material is said to be **anisotropic**.

Ovalbumin seems to be a transparent crystal. When rotated through 360^0 it is found to be isotropic (Uniform in all direction) that is with analyser-in, the object was invisible, for all orientation of the microscope stage from 0^0 to 360^0. Crystals have similar properties in all directions.

When beam of polarised light passed through the crystals of Zinc-Ovalbumin complex, at a specific orientation the crystal allows the passage of light through it and seems to be bright (Fig 22). But when the stage is rotated, the bright appearance change and after rotation through 45^0 it seems to be dark (Fig 25) since it does not allow the passage of light. Again after 45^0 rotations it seems to be bright (Fig 23). At every 45^0 rotation when polarized light passed through the crystal it appears to be bright and dark alternatively. Since the crystals of Zinc-Ovalbumin complex shows this phenomena it is said to be anisotropic.

Examination of micrographs of ovalbumin and the metal complex indicates very definite change in property. Albumin crystals are found to be isotropic. They are visible under ordinary light, but they are not visible under polarised light. The Zn complex crystallised over microscope slide in form of crystals of definite shape. Very beautiful hexagonal crystals are formed. These crystals are clearly anisotropic, and seen under polarised light.

The definite change in optical property also suggests the formation of Zn-Ovalbumin complex.

CONCLUSION

In this project work, a crude but simple method for preparing Zinc-Albumin complex is obtained. The identity of the conjugate is suggested by its properties like SEM image ,I R Spectrum and U V spectrum which are distinctly different from corresponding observations on albumin crystallized from ovalbumin solution.

We studied the optical properties of the complex by taking micrographs using polarizing microscope and noted the peculiarities of complex in various stages.

Zinc albumin complex formed by mixing the respective solutions in slightly varying proportions show formation of hexagonal crystals. Such crystals are obtained both in presence and absence of surfactant. In presence of the surfactant, large number of smaller crystals are formed.

We have prepared Zinc-Albumin complex with Curcumin loaded on it by simple mixing of aqueous solutions in specific preparation on glass plate and drying .But only very small amount of needle like crystal is obtained. The SEM image of curcumin conjugate also shows only small change in the albumin surface.We have not attempted any method for the purification of the prepared sample.

But the SEM image of conjugate with the drug 5-fluoro uracil shows thorough morphology change over the surface of Albumin solid .

Further investigation of the properties of the complex material produced is required to find out its applications. Generally such materials are used for drug loading and controlled releasing. Characterisation of the curcumin loaded albumin zinc complex can be investigated .Effective loading and releasing of curcumin on a non toxic substrate can improve the bioavailability of curcumin and its use as a drug.

Particle size anslysis is done for metal albumin conjugates. This is only a preliminary level reporting of formation of particles of nano level diameter. Further work has to be done to optimise the preparation technique and also to obtain particles of definite size. Particles of this dimension can be put to several applications like that for drug loading and releasing.

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