GENIPIN CROSSLINKED GELATIN-GUARGUM BASED PHASE SEPARATED HYDROGEL FOR DRUG DELIVERY AND TISSUE ENGINEERING APPLICATION

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CERTIFICATE

This is to certify that the thesis entitled **"GENIPIN CROSSLINKED GELATIN-GUARGUM BASED PHASE SEPARATED HYDROGEL FOR DRUG DELIVERY AND TISSUE ENGINEERING APPLICATION"** is a record of the bonafide work done by SUBRAT PRUSETH (111BT0535) and BAIKUNTHA BEHERA (111BT0586) which is submitted for partial fulfilment of the requirements for the degree of Bachelor of Technology (B.Tech) in Biotechnology Engineering at National Institute of Technology, Rourkela. To the best of my knowledge, the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any Degree or Diploma.

Place: NIT Rourkela

Date: 11th May, 2015

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ABSTRACT

The rising cost of development of new drug has made researchers to look for an alternative way. In recent years researchers have given a lot of importance to increase the effectiveness of the existing drugs. No other thing affects the potency of the drug as its drug delivery system does. A lot of research is being going on to develop a novel drug delivery system. A lot of research has been done on different combination of guar gum and other substances as a drug delivery system and most of them have been done in India. Not only does guar gum is being used in food items extensively, it is also thought to have medicinal properties and optimal properties to be a drug delivery system. Guam gum is abundantly and cheaply available; with 80% of total world production is from India. In this study we have researched the possibility of using Genipin cross linked Guar gum-Gelatin phase separated hydrogels. We have done MTT assay with MG-63 cells to check cell viability, drug release test with Metronidazole, and hemocompatibility with human blood to check bio compatibility.

INTRODUCTION

Developing a new drug is always associated with two factors: time and cost. Since it consumes lot of time and the process is also very expensive. In general a drug development cost around 2 billion dollars. On an average it takes 12 years for the new drug to enter the market from the lab. The prices for drug development are going on increasing and the cost is transferred to the customers. The prices of drug have increased a lot and it's now a luxury that the common man cannot afford. The no of drugs being developed or discovered have declined in the last few years.

Number	1990-1994	1995-1999	2000-2004	2005-2009
Total	215	207	162	146
Average per year	43	41	31	29

Table 1: - Number of new biological entities(1990-2009)

A better alternative is to increase the potency of the old conventional drugs. The other alternative methods which have been pursued vigorously are drug delivery at slow rate, controlled delivery, targeted delivery and better body distribution. Great strides have been made in this field in the past 30 years.

Challenges in drug delivery

Current methods have their specific limitations which are being addressed by the research community. Common example is that the drugs have low half circulatory life and it gets degraded before getting to the targeted sites, the drugs won't get to the targeted site, the drug won't be delivered in a controlled manner or they are not distributed in the body [1].

Different methods of drug delivery system

A number of different drug delivery systems have been developed and are being researched upon in the recent years. This drug delivery system may be external or internal. Some examples of external drug delivery systems are ointments and drug patches. Examples of internal drug delivery systems include lipid, protein and polymeric technologies. Normally all these delivery systems work by encapsulating or entrapping the drug and releasing at their required site [2].

Properties for a drug delivery system

There are few properties that that are essential for drug delivery systems. They should be biodegradable, biocompatible, non toxic, it shouldn't cause immunogenic reaction, it shouldn't react with the drug, protect the drug from getting degraded, deliver the drug to the targeted site of release, release drug in a control manner.

Guar gum based drug delivery system

Recently a lot of studies and research have been done upon using guar gum as a drug delivery system. Guar gum is a biodegradable, biocompatible, nontoxic, non-irritant material, widely available and cheap. Guar gums are produced from guar beans and 80% of world total production of guar beans comes from India. It has been known to be used in various food items. It is a non-ionic polysaccharide and has various desirable properties for being used a drug delivery system [3]. It can be chemically modified to change its properties and can be used according to our requirements. It is been widely used in various industries like pharmaceutical, textile, paper, food, petrochemical, etc.

Problems faced with guar gum

Even though guar gum has many desirable properties it comes with its own sets of challenges. Being a polysaccharide it is highly susceptible to microbial attack. It has an uncontrollable rate of viscosity, uncontrollable rate of hydration, high swelling characteristics; instability of its solution for a long time restricts its use in pharmaceutical industries.

Solutions

Being a polysaccharide guar gum can be chemically modified according to our requirements. Cross linking the aqueous solution of guar gum and gelatin with genipin produces a phase separated hydro gel [4]. The cross linking solidifies the solution and also increases the mechanical properties of the solution. It also decreases the swelling properties of the guar gum.

Hydrogels

Hydrogels are generally the polymer networks that have been crosslinked physically or chemically and which can absorb large amount of water. They can be classified into different groups based on various parameters such as preparation methodology and on their mechanical, structural and biological characteristics [5]. The properties of gels such as gel strength, degradability, biocompatibility, swelling, porosity, and many other properties are important during the design of a hydrogels and which can be controlled through cross linking method and other parameters such as down streaming processes and conditions like temperature, pressure and sterility.

Phase Separation

In general, phase separation results in separation of polymer solution into two distinguish layers. Even though, the effect is non-desired, the phase separated solutions show some peculiar rheological properties which attracted the attention of research community and found their application in biomedical field . Phase separation might be due to difference in solubility or in Gibbs free energy which might have resulted in immiscible nature of the solutions [6]. The stability of the phase separated gels may vary from few hours to few weeks and the same depends on the nature of the polymer and its solubility. The stability of the gels can be increased by physical or chemical cross linking which can prolong the stability of the gels.

Materials for Hydrogel Preparation:

In common there are two main categories of hydro gels: (i) gels that form naturally such as gelatin, collagen, guar-gum, alginate and fibrin, here all the components are polymers of natural origin and (ii) synthetic hydrogels [7]. Synthetic hydrogels are the gels that comprises of wide range of gels that has been formed from pure synthetic polymers or polymers that have been derived or modified from natural polymers or synthetically produced. In this study we will be making naturally forming hydrogel.

Applications

Hydrogels of this type can find its application in both drug delivery as well as tissue engineering. In addition, the need of injectable and biodegradable gels is in great demand for biomedical applications. As we mentioned earlier, the physical property of the gels can be manipulated through processing parameters and which can be made to meet the properties of scaffold to match with those of native tissue [8]. Hydrogels

can also find their application in tissue recovery after tumour or trauma evacuation. Various specialists have concentrated on the mix of injectable hydrogels and biodegradable microspheres for controlled drug delivery in tissue engineering [9].

LITRETURE REVIEW

During the past few decades, hydrogels have been in use for biomedical applications such as in vivo controlled drug delivery systems, tissue implants, organ transplant and, in situ tissue scaffolds. The current study deals with the development of Genipin crosslinked phase-parated hydrogel for both controlled drug delivery and biomedical tissue engineering applications. The hydrogels were prepared by varying proportion of gelatin and guargum. A natural crosslinker "Genipin" is added to cross links with gelatin. Hydrogels are type of gels where the solvent is water. All natural biopolymers used here are water solvable molecules. First of all coming to the definition of gel, it is defined as the non-fluidic colloidal material forming polymer network that is expanded throughout its whole volume by a fluid. These gels are may be soft or hard depending on the composition or ratio of chemicals taken having different properties [10]. It is a 3-Dimensional crosslinked network within the liquid. The crosslinking inside the liquid that gives a gel, gels are scattering particles of a fluid inside a strong in which the robust is the consistent stage and the fluid is the spasmodic stage.

Gels are of 3 type's i.e. hydro gels, organo gels and xerogels. Hydrogels is a system of polymer chains that are hydrophilic, colloidal gel where water is the scattering medium. Hydrogels are highly permeable natural or synthetic polymer networks [11]. Due to their high water content they also poses high degree of flexibility in similar to native tissue. Basically there are three types of phase separated systems that are water-in-water, water-in-oil and oil-in-water emulsions. Here we are taking water-n-water emulsion which consists of two water solvable molecule having different properties [12]. Using this type if emulsion system we can get a phase separated hydro gel which consists of droplets of water solvated molecules in another

continuous aqueous solution. So it has two type of phase i.e. droplet and continuous phase. The molecules in the both phase are entirely water soluble. So when the both phase are mixed water droplets containing predominantly one component (gelatin) are dispersed in water solution containing another component (guar gum).

Guar gum

Guar gum is a natural non-ionic polysaccharide that is abundantly found. It is derived from the ground endosperm of Guar beans (*Cyamopsistetragonolobus*) which is used as a green vegetable. Guar beans are native to Indian subcontinent and about 80% production is being done in India. Guar gum is commonly used in food as a binder, thickener and stabiliser. Other than food industries guar gum has found its application in other industries such as textile, paper, explosive, pharmaceutical, cosmetics, hydraulic fracturing, mining, etc. It is white to yellowish white, nearly odourless powder with a bland taste. It is practically insoluble in organic solvents. In cold or hot water, guar gum disperses and swells almost immediately to form a highly viscous thixotropic solution [13]. It is shown to have many medical properties and it has been used as an agent for weight loss. Theoretically, guar gum decreases appetite by providing a feeling of fullness. It is also used in diabetic diets, can decrease the cholesterol level, relieving constipation, relieve abdominal pain, etc. Guar gum contains about 80 % of galactomannan. The backbone is a linear chain of β 1,4-linked mannose residues to which galactose residues are 1,6-linked at every second mannose, forming short side-branches.

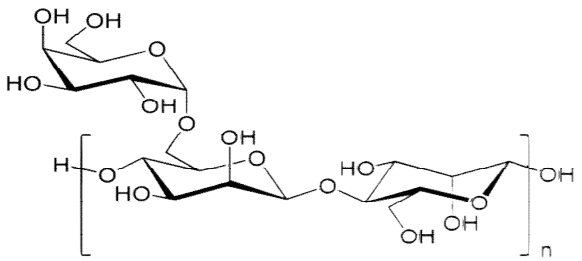


Fig 1: - molecular structure of Guar gum.

The mannose to galactose ratio has been found to vary from 2:1 to 1.8:1. The average molecular weight of guar gum has been reported as $1\text{-}2x10^6$. Beta linkage between the monomers makes the guar gum as rigid rod like polymer.

In water guar gum is non-ionic and hydro colloidal. It is soluble in cold water, hydrating quickly to produce viscous pseudo plastic solutions that although shear-thinning generally have greater low-shear viscosity than other hydrocolloids. It is not affected by ionic strength or pH, but will degrade at extreme pH and temperature [14]. It remains stable in solution over pH range 5-7. It is a nontoxic, biodegradable, biocompatible substance, which is cheaply and abundantly available. It can be chemically modified to our requirements. These properties make guar gum an excellent choice for a drug delivery system. However, the aqueous solution of guar gum has an uncontrollable rate of viscosity and hydrating. The solution tends to degrade in the time period of 1-2 days, loses its viscosity. The solution is highly susceptible to microbial attack. This affects its effectiveness as a drug delivery system.

Gelatin:

Gelatin is flavourless, translucent, colourless, purified protein derived from collagens obtained from various animal by-products. It is produced as a by-product of meat and

leather industries. Sources of gelatin are pork, pork skins, swim bladders of fish, horses, and split cattle hides or cattle bones. Depending upon the source, gelatin might differ in their physical properties. It has various uses in many industries such as food, pharmaceutical, photography, nutraceuticals, cosmetics. It is commonly used as a gelling agent in food items.

When gelatin is placed in liquid, the molecules swell and then dissolve, and the chains separate. After cooling, they re-form as tightly as before. Gelatin solution solidifies below 35^{o}C, which is a 100% thermo-reversible gel. Gelatin is a polyampholyte which means it has both cationic and anionic groups along with hydrophobic groups. These groups are present in the approximate ratio of 1:1:1. Its molecule is ~ 13% positively charged, ~ 12% negatively charged, ~ 11% of the chain hydrophobic in nature [15]. Glycine comprises about one-third of the chain, proline the other one-third and the rest are residues. The typical amount of amino acids present in gelatin, the percentage may vary depending upon the source of gelatin.

Alanine	8.9%	Isoleucine	1.5%
Arginine	7.8%	Leucine	3.3%
Aspertic acid	6.0%	Lycine	3.5%
Glutamic acid	10.0%	Methionine	0.7%
Glycine	21.4%	Phenylalanine	2.4%
Histidine	0.8%	Proline	12.4%
Hydroxylysine	1.0%	Serine	3.6%
Hydroxyproline	11.9%	Threonine	2.1%
Valine	2.2%	Tyrosine	0.5%

Table 2 : Typical amino acids present in gelatin.

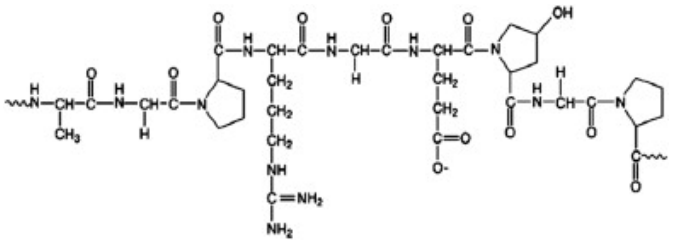


Fig 2: - Molecular structure of Gelatin.

Genipin

Genipin is an aglycone derived from an iridoid glycoside called Geniposide present in the fruit of *Gardenia jasmindides*. It is bi-functional natural crosslinker for gelatin,proteins, chitosan, and collagen. Upon crosslinking with gelatin, it produces a blue coloured fluorescent hydrogel.

Reaction mechanism:

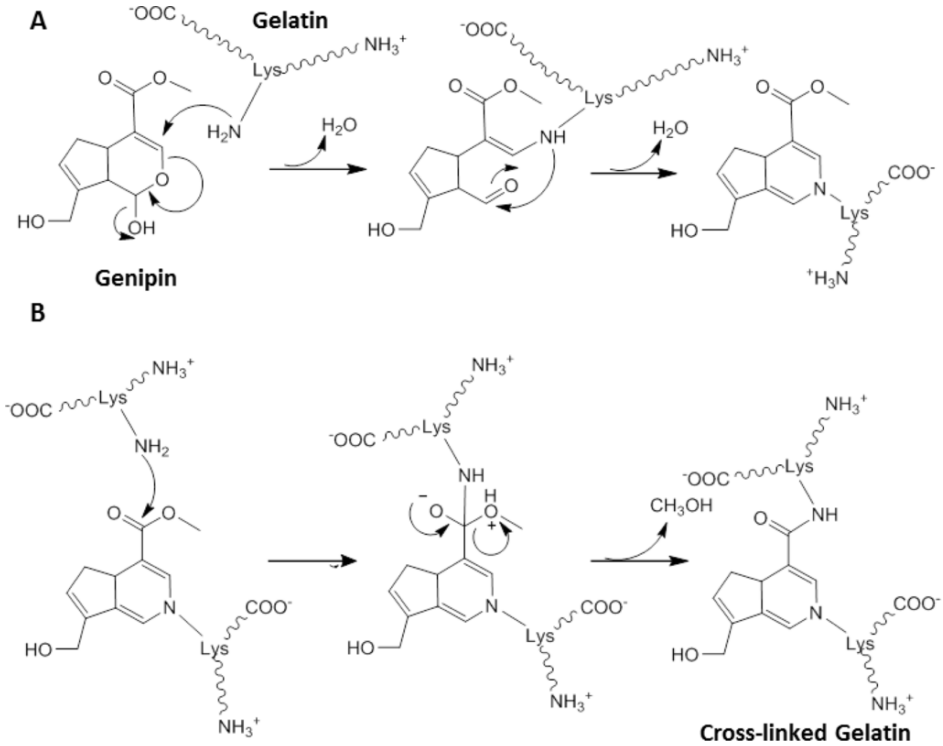


Fig 3: - Reaction mechanism of Gelatin and Genipin.

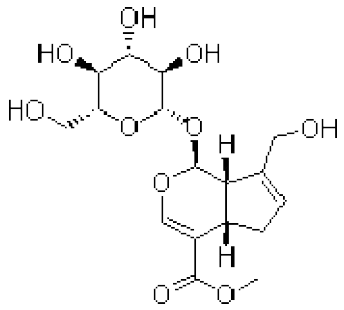


Fig 4: - Molecular structure of Genipin.

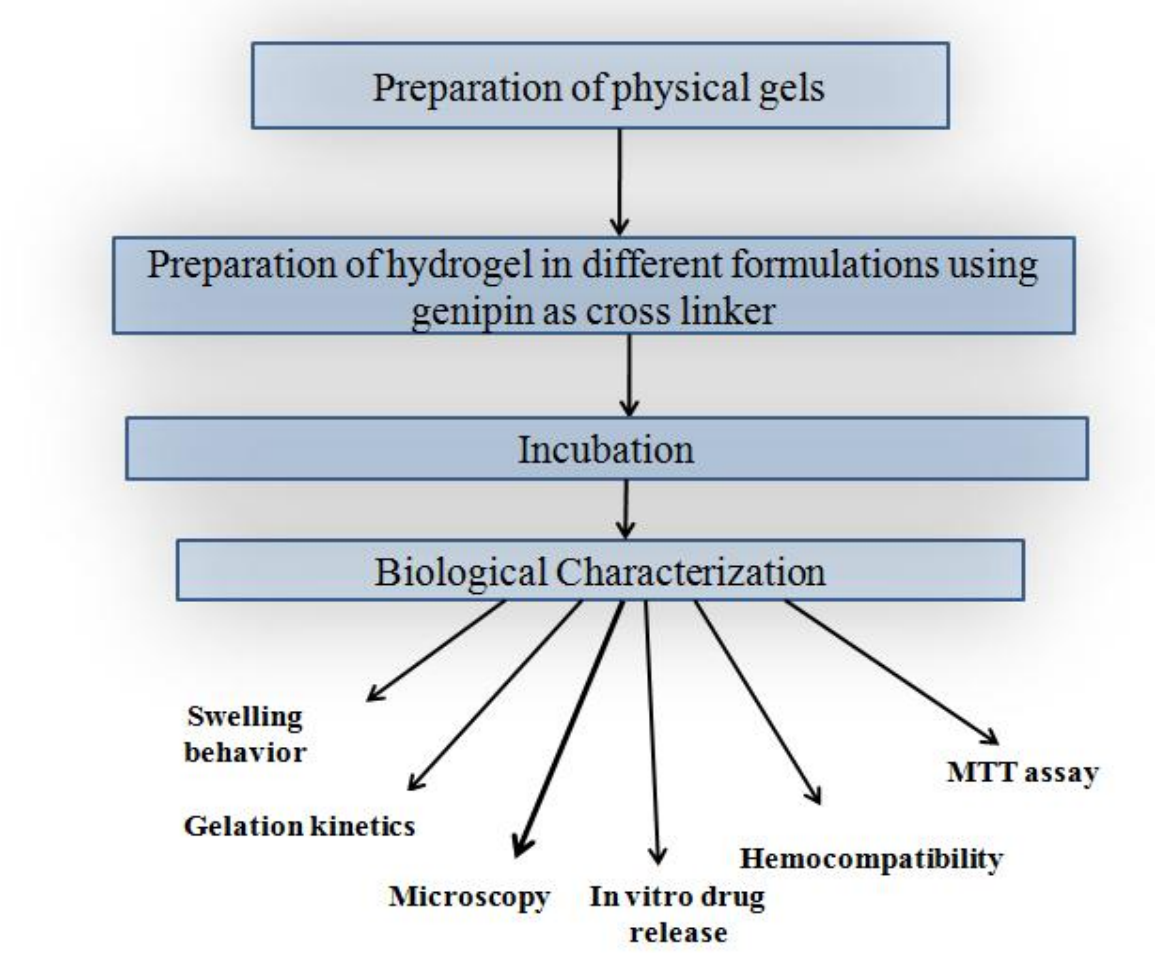
Genipin has a very low acute toxicity, with LD_{50} i.v. 382 mg/kg in mice, making it excellent crosslinker of biopolymers for tissue engineering purposes. It has better stability and biocompatibility than Glutaraldehyde and it might replace glutaraldehyde in the future for pharmaceutical purposes. It is soluble in Methanol, Ethanol, Acetone, and slightly soluble in water.

Here we use genipin a natural bio molecule as a crosslinker for preparation of hydrogel. Crosslinkers are the inter connection between polymer chains. They may be covalent bonds or ionic bonds or co-ordinate bonds. When a crosslinker is used in science field it usually promotes a difference in the physical properties of the polymer. Crosslinkers are used both in synthetic polymer chemistry and biological sciences [16]. For the better result than the former sample we use crosslinkers. Here are some advantage of genipin for which it can be preferably used for drug delivery and tissue engineering applications.

OBJECTIVE:

- To prepare a nontoxic biodegradable and biocompatible hydrogel based drug delivery system.
- For a controlled drug release, different formulations of genipin cross-linked guar gum gelatin hydrogels were studied.

WORK PLAN



EXPERIMENTAL

Materials

Gelatin, Guargum, and were purchased from Himedia, Mumbai, India. Metronidazole (MZ) was a kind gift from Aarti drugs, India. Genipin was procured from challenge bioproducts Co., Taiwan. Dulbecco's Minimal Essential Media (DMEM), Dulbecco's Phosphate Buffer Saline (DPBS), Trypsin-EDTA solution, Fetal Bovine Serum, Antibiotic-Antimycotic solution, and MTT assay kit were purchased from Hi-media, India.

Methods

Preparation of physical gels:

Guargum (2%) stock solution was prepared by taking 30 ml of sterile distilled water (DW) in a beaker. Add the guargum powder slowly to distilled water in a constant stirring condition. The rpm of magnetic stirrer was set to be 500 and we kept the sample for 4 hour. After formation of gel we centrifuged it at 7000 rpm for duration of 30 minutes for removal of unwanted particles and to get a homogeneous mixture of guargum. As guargum is very prone to microbial contamination so we should store it in refrigeration for further use but not more than 2 days as the viscosity decreases.

Gelatin (20%) stock was prepared by taking 30 ml of sterile distilled water (DW) in a sterile beaker. Add the gelatin powder slowly to the beaker which is on a constant stirring condition at 400 rpm. As the gelatin is a protein it won't get dissolve, so we kept it in the hot air over covering with aluminium foil, maintaining 50-60°c for 15 minutes.

Both the solution of guar gum and gelatin were weighed and poured into a test tube. The solutions were then mixed using vortexer. After mixing the solution were kept inside an incubator for 12 hrs.

Preparation of genipin crosslinked hydrogels:

The genipin (15mM) crosslinker solution used here was prepared taking weighted amount of genipin powder in an eppendorf tube and 250 µL of DMSO (solubilisation solution) was added [17]. Shake it for mixing it properly and after that dilute it to 700 µL with distilled water. We are adding DMSO here as it is less Cytotoxic in nature.

The phase separated hydro gels were prepared by varying the compositions of the gelatin (20%), guargum (2%) solutions and genipin (15mM) is added in same amount to all the formulations as shown below (Table. I). First we added guargum to the testube and then we added genipin solution of 100 µL to each of the varied formulations. We vortexed the mixture to get a homogeneous mixture of genipin throughout the sample. After that we added the required amount of prepared gelatin solution as per formulations. Then again after adding gelatin the total solution was mixed using a vortexer. The mixed solution was kept in an incubator for preparation of solidified hydrogel.

Sl. no	Sample Name	Guargum (2%)	Gelatin (20%)
1	GG-30	30	70
2	GG-40	40	60
3	GG-50	50	50
4	GG-60	60	40

5	GG-70	70	30
6	GG-80	80	20
7	GELATIN	0	100

Table 3 : Formulation of gelatin and guargum varying concentration

As gelatin is not so viscous it may form air bubbles during vortexing, so to remove the bubbles we kept it in the dessicator for 15-20 min. The crosslinking activity needs some environmental condition, so we kept it in the incubator maintaining at a temperature of 37°c for 24 hours.

Microscopic Evaluation of hydrogels

Phase contrast microscopy helps understanding the arrangement of the protein phase and the carbohydrate phase in a phase separated formulation. We prepared wells(**Fig 4**) by cutting the base of tips and attached them to cover slits using PDMS solution. We pour 300µL of sample to each of triplicate wells [18]. And we tilted the wells to cover the whole surface with samples, so as to get a very thin of smear. We put the samples in a box in moist condition and in sealed condition.

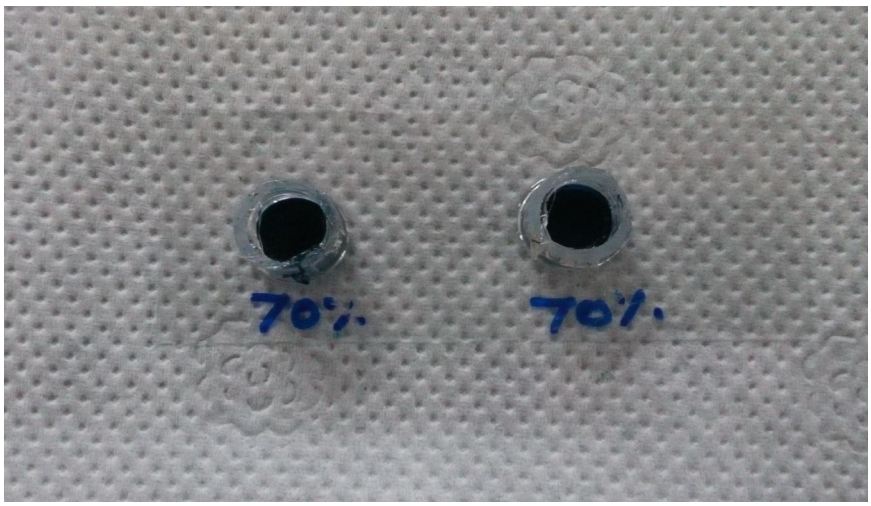


Fig 5: - Wells for microscopy.

Gelation kinetics of hydrogels

The time duration required for formation of gel after addition of crosslinker was studied. This was performed by adding the crosslinker into the aqueous solution of different formulations of gelatin and guar gum [27] While in liquid form the gels were constantly observed till they solidified.

Swelling studies of hydrogels

The swelling behaviour of our gel was investigated at room temperature by exposing them to PBS solution. A known weight of gel tablet was placed in PBS solution for 24 hour period. The weight of the gel was determined blotting the gel surface with tissue paper to remove excess surface water then weighed immediately [19]. The percentage of water absorption (W_{sw}) by the tablet (gel) was calculated from the following expression.

$$\mathbf{W}_{SW} = \frac{\mathbf{W}\,final - \mathbf{W}\,initial}{\boldsymbol{W}\,initial} \times 100$$

Where, **W** final = the weight of gel tablet after immersing in PBS

W initial = the initial weight of the gel tablet before immersing into PBS

W sw = it represents the degree of swelling

In Vitro Drug Release Studies of hydrogels

The drug used here is Metronidazole. Before performing this experiment we should stop the cross linking activity of genipin using 0.3M Glycine solution. We cut the preformed gels to get a uniform tablet shape having same weight, for which the concentration of drug will be same in all pieces of hydro gel. The release of Metronidazole from the Metronidazole-loaded samples was performed *in vitro* using triplicate of samples in 24 well plates, as our sample is so rigid enough we directly

dipped the sample in PBS [20]. The each compartment of 24 wellplate contained 3 mL of phosphate buffer saline (pH 7.2). At a regular interval of time we replace the entire amount of PBS with fresh PBS. The replaced release media was observed at 321 nm using UV–Vis spectrophotometer (UV 3200 Double Beam; Labindia, Thane (West), Maharashtra, India) [28]. The study was conducted for 12 h.

Hemocompatibility study of hydrogels

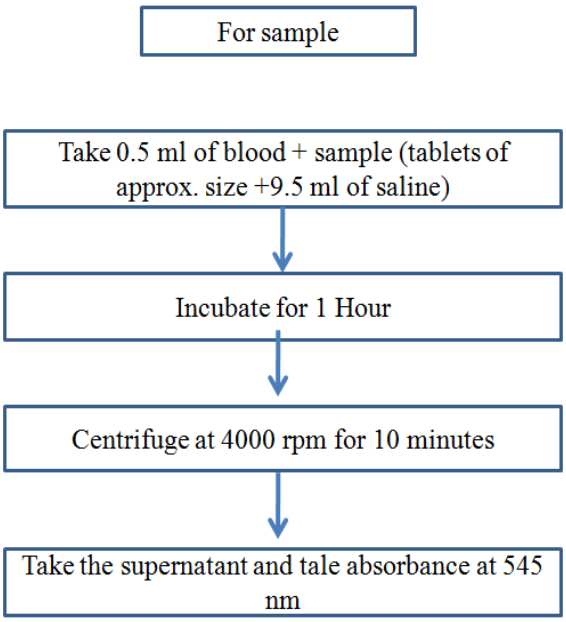
When biomaterials like hydrogels come directly in contact with human blood, it induces some reaction i.e. between blood protein, cells, and biomaterial. The rate of this reaction depends on the surface topography, hydrophilicity, charged particles and the state of gel. This experiment was done to observe biomaterial and blood cells interaction which depends on the use of realistic incubation setup with well defined parameter [21]. The hemocompatibility study was done by using human blood (A +ve).

Protocol

- Cut the gels into regular pieces having approximately same weight, so that the surface area will remain approximately same.
- Prepare 0.9% of saline water and dip the entire triplicate tablet sampled in to the saline for overnight.
- Take 3.8% of tris sodium citrate as per required amount of blood in a falcon tube before collecting blood because it prevents the blood from clotting.
- Prepare the stock solution of blood i.e. (blood: saline :: 8:10).
- Prepare 0.1 M of HCl solution for positive control.

Sample preparation

For hydrogel samples the procedure is mentioned as below in the chart. We prepared two controls here, one is positive control and another one is negative control. In both controls no sample is present [29]. In positive control we took 0.5mL of stock blood and 0.5mL of 0.1M HCl solution. But in negative control 0.5mL of saline was added to 0.5mL of stock blood. Both the controls were diluted to 10mL with 0.9% of saline water.



Calculation:

$$\% \text{ Hemolysis} = \frac{\text{O.D Sample} - \text{O.D} - \text{ve control}}{O.D + ve\ control - O.D - ve\ control} \times 100$$

Where O.D sample = Absorbance of the test sample

O.D –ve control = Absorbance of positive control

O.D +ve control = Absorbance of negative control

Cell viability assay of hydrogels

The cells MG63 were collected from NCC Pune laboratory, and sub cultured in our laboratory for further use. The cells were developed in the following steps. Cell proliferation of the samples was studied using MTT Assay on MG-63 cell line (from NCCS, Pune, India). The cells were maintained in complete DMEM media (10% FBS and 1% antibiotic solution) at 37°C, 5% CO_2. The cells were passaged and 1 x 10^4 cells/well were added in 96 well plate and incubated for 24 hrs for the cells to adhere properly [22]. After 24 hours of incubation, the cells were treated with HAP samples at a concentration of 100µg/ml and then again incubated. After 1^{st}, 3^{rd} and 5^{th}day, MTT assay was carried out by adding 100µl of MTT media (MTT reagent and DMEM complete media in the ratio 1:10) and incubated for 4 hrs. After that, MTT media were discarded and 100µl of DMSO was added to stop the reaction and to dissolve the formation crystals. The absorbance was then measured at 595nm.

Cell culture

Initially cells are in T-25 flask

↓ **Remove spent media**

Add 1 ml of trypsin + EDTA solution

↓ **Wash and remove the solution**

Add 2 ml of trypsin + EDTA

↓ **Incubate for 5 minute**

Add 2ml of fresh media DMEM (complete)

↓

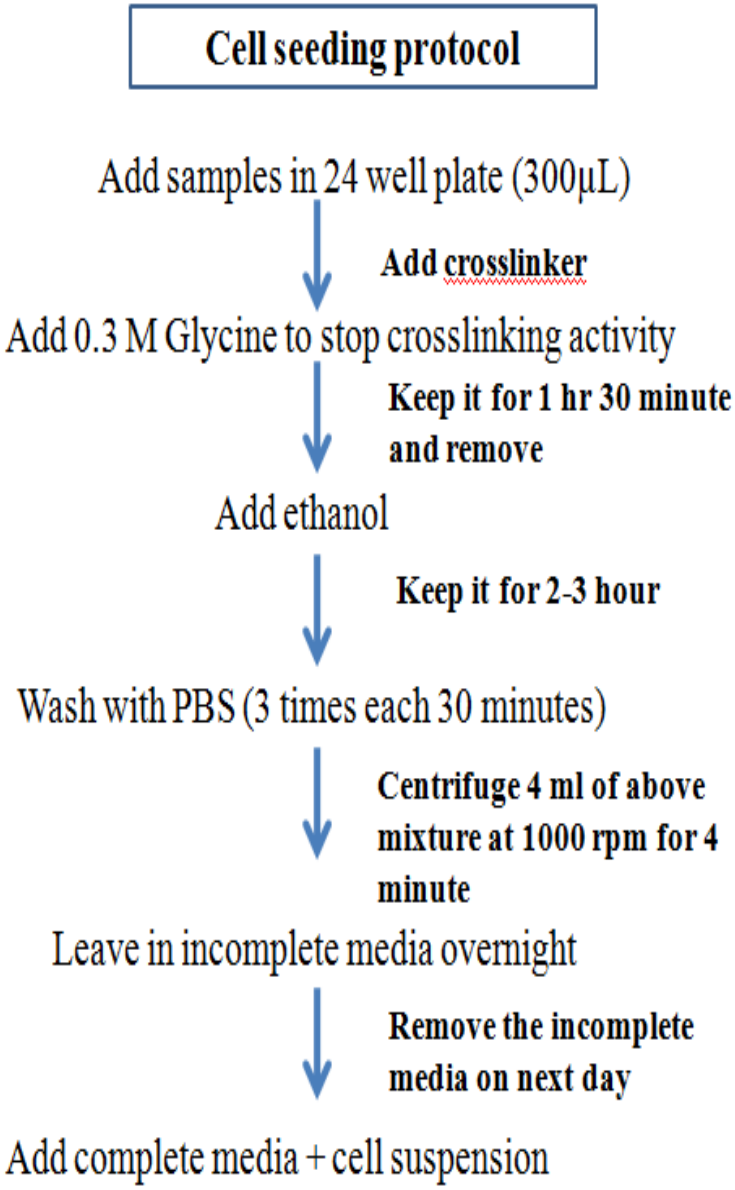
Centrifuge 4 ml of above mixture at 1000 rpm for 4 minute

↓ **Discard the supernatant**

Add 300 µL of media on to a hemocytometer

↓

Calculate the no of cells and also dilute to make it 4×10^4 cells per ml. As we are using 24 well plate method.


Cell seeding protocol
Add samples in 24 well plate (300µL)
Add crosslinker
Add 0.3 M Glycine to stop crosslinking activity
Keep it for 1 hr 30 minute and remove
Add ethanol
Keep it for 2-3 hour
Wash with PBS (3 times each 30 minutes)
Centrifuge 4 ml of above mixture at 1000 rpm for 4 minute
Leave in incomplete media overnight
Remove the incomplete media on next day
Add complete media + cell suspension

RESULTS AND DISCUSSION

Preparation of physical hydrogels

The prepared hydrogels of different formulation of guargum and gelatin were optimized. The concentration of guargum was changed from 30%-80% where the concentration of gelatin was varied from 70%-20% respectively. At low concentration of guargum stable gels were formed but increasing concentration of guargum phase separated emulsion gels were observed. The emulsion formulations were confirmed by tilting the testube, as the emulsions start flowing due to gravitational force. Emulsion gels were formed above 50% concentration of guargum. The lower concentration of guargum shows stable and solid gels which were confirmed by tube inversion test method. And the length of phase separation was calculated using a scale. The varied formulation plays an important role in formation of gels or emulsions. In lower concentration of guargum the gels were formed without crosslinker because of formation of crystalline structure. That means the continuous phase get trapped in the droplet phase. The formation of gels and emulsions are showed in the following figure.

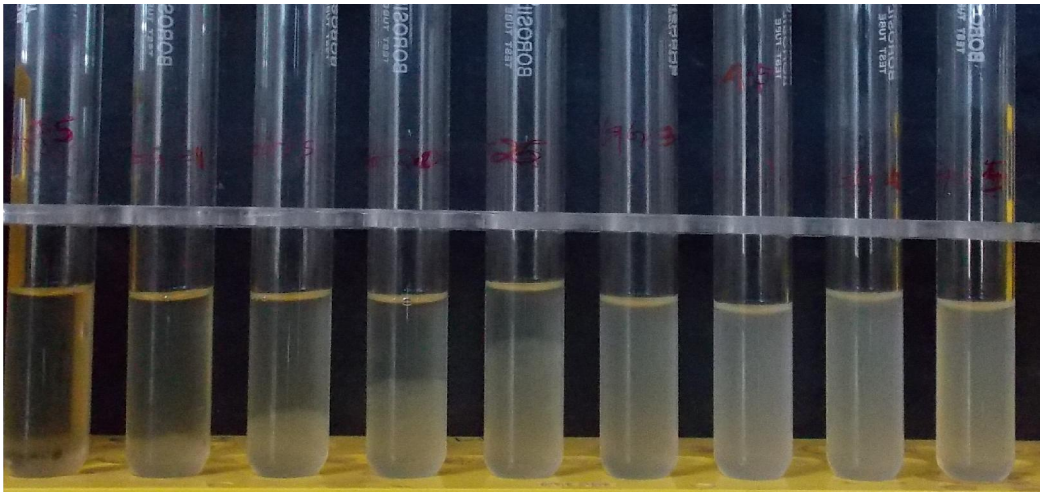


Fig 6:- Phase separated and water-in-water emulsion hydrogel.

As shown in **fig.5** increasing the concentration of guargum the phase separation increased till 50% w/w of guargum and gelatin, after which the phase separation was not visible. We performed tube inversion method to detect whether the composed formulations are gel or emulsion.

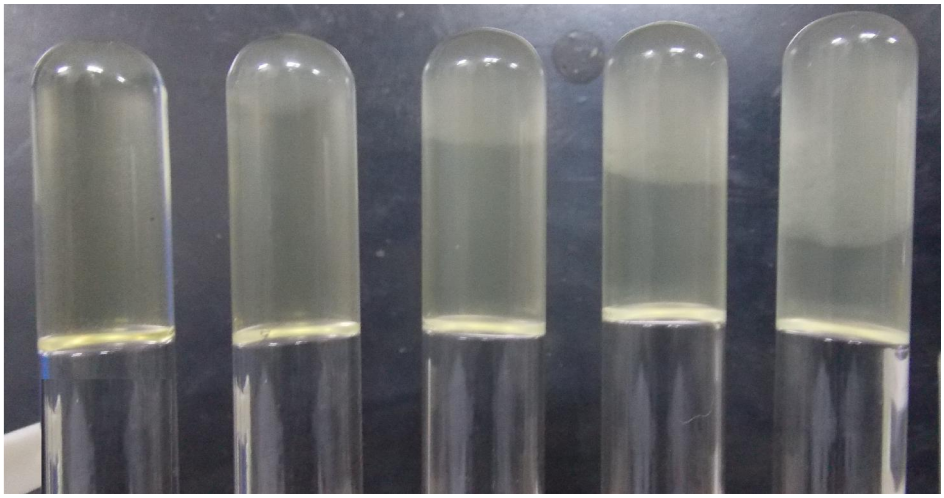


Fig 7: - Inverted tube test of phase separated hydrogel.

As shown in **Fig.6** with increase in guargum concentration till 50% w/w, we obtained a clear visible phase separated gel, Which was clearly visible in tube inversion test? There was no flow during the tube inversion test, so we can conclude that gel was formed.

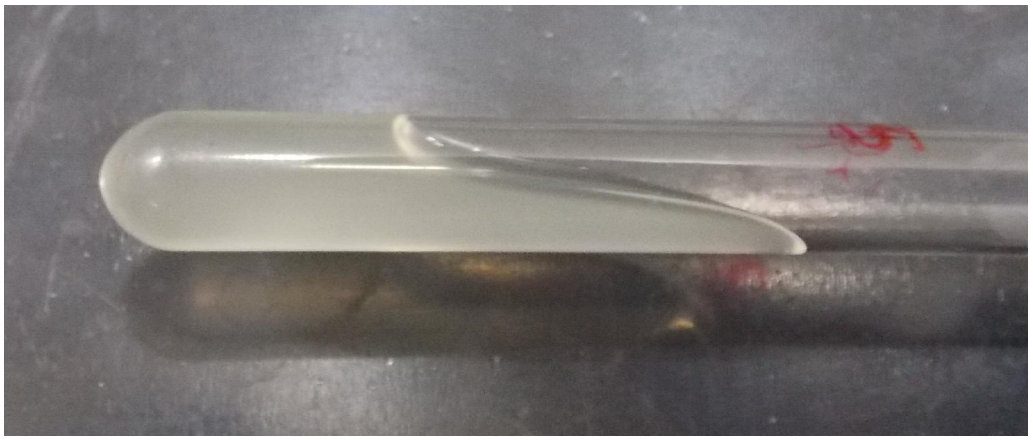


Fig 8: - water-in-water emulsions.

In **Fig.7** though the concentration of guargum increased more tha 50% w/w, it could not assemble the protein (gelatin). So emulsion was formed which was also phase separated but it was not broadly clear.

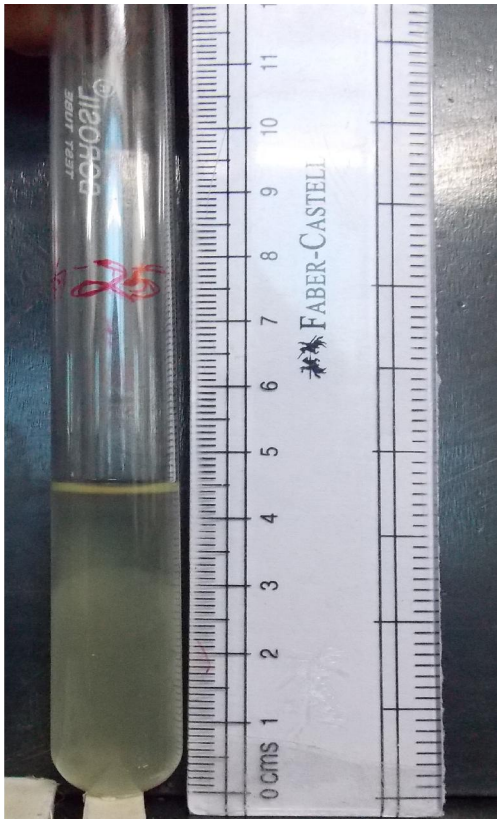


Fig 9: - Measurement of length of phase separation

In **Fig.8** the length of phase separation was measured. According to the concentration variation the length of the phase separation also changes. When we increase the concentration of guargum the length of separation also increases. We measured the length of the separation using a scale. That separation explains that how much immiscible the system is. Due to immiscibility and the different solubility two solutions don't mix properly, though we found a clear and homogeneous solution after vortexing .The detail length measurement that observed in naked eye is mentioned in the **Table.4.**

Sample proportion(in gm)		Tube inversion test	Water-in-water Emulsion	Phase separated hydrogel	Length of phase separation cm)	
Guargum solution (2%)	Gelatin solution (10%)				Phase separation	Total length
0.5	4.5	No flow	No	Yes	0.5	4.1
1.0	4.0	No flow	No	Yes	1.0	4.1
1.5	3.5	No flow	No	Yes	1.6	4.1
2.0	3.0	No flow	No	Yes	2.3	4.1
2.5	2.5	Slight flow	No	Yes	3.2	4.4
3.0	2.0	Flow	Emulsion	No	No separation	4.1
3.5	1.5	Flow	Emulsion	No	No separation	4.1
4.0	1.0	Flow	Emulsion	No	No separation	4.3
4.5	0.5	Flow	Emulsion	No	No separation	4.4

Table.4: Tabulation for different characteristics of prepared phase separated hydrogel without genipin.

Genipin crosslinked hydrogels were made in a test tube and there phase separation length was observed. In comparison to non crosslinked gels, all the formulations of crosslinked gels had phase separated.

Fig 10: - Phase separation in genipin crosslinked hydrogels

Sample	Length of phase separation	Total length	% of total length
30%	1.4	2.9	48.27
40%	1.3	2.6	50.00
50%	1.0	2.2	45.45
60%	0.9	2.5	36.00
70%	1.0	2.5	40.00
80%	1.4	2.6	53.84
Gelatin	0	2.7	0

Table 5: Tabulation for different characteristics of prepared phase separated hydrogel after crosslinking of genipin.

Gelation kinetics of hydrogels

It was observed that the gels having lower concentration of guargum (GG-30, GG-40 and gelatin) were solidified at a time period of 3 hour. The gels having guargum concentration of GG-50 and GG-60 were solidified after a time period of 4 hour and rest formulations were solidified at a time interval of 5 hours. From the above observations we can conclude that with increasing concentration of guargum the time required for sample to solidify increased. One more thing we can assume that the crosslinking activity of genipin starts after 2 hour of mixing and the saturation point meets at 5 hour. The detail report is motioned in the **Table 6.**

Time (hr) sample	**0**	**1**	**2**	**3**	**4**	**5**
30%	Not Formed	Not Formed	Not Formed	Formed	Formed	Formed
40%	Not Formed	Not Formed	Not Formed	Formed	Formed	Formed
50%	Not Formed	Not Formed	Not Formed	Not Formed	Formed	Formed
60%	Not Formed	Not Formed	Not Formed	Not Formed	Formed	Formed
70%	Not Formed	Not Formed	Not Formed	Not Formed	Not Formed	Formed
80%	Not Formed	Not Formed	Not Formed	Not Formed	Not Formed	Formed
Gelatin	Not Formed	Not Formed	Not Formed	Not Formed	Not Formed	Formed

Table 6: Gelation kinetics study at different interval till formation of solid gel.

Microscopic study of hydrogels

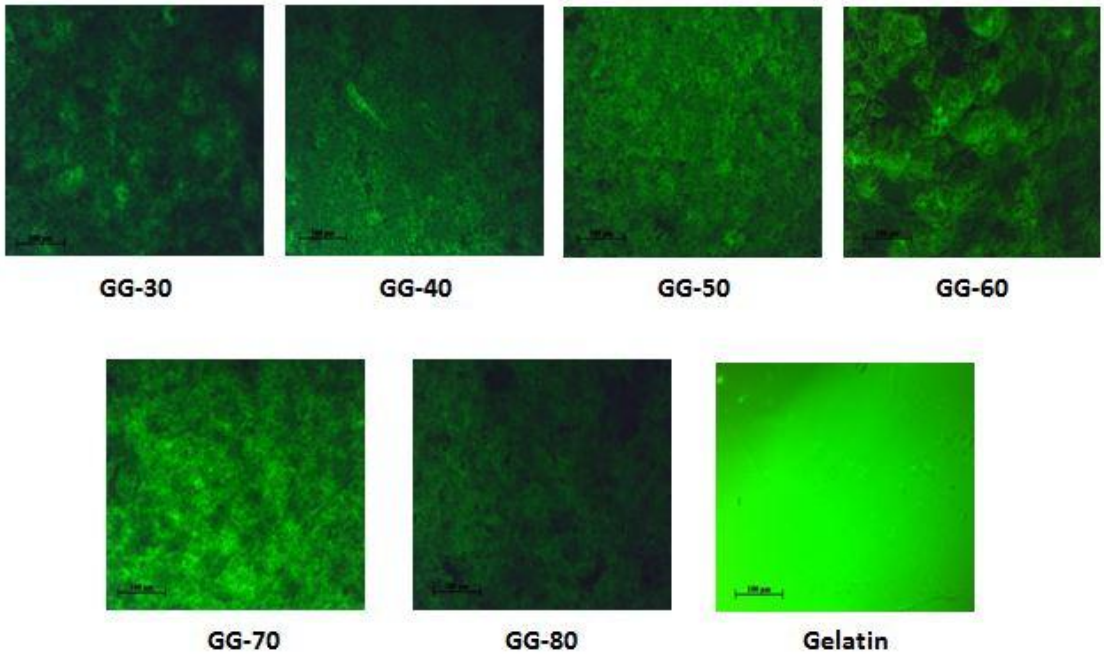


Fig 11: - Phase contrast microscopy of hydrogels

Swelling studies of hydrogels

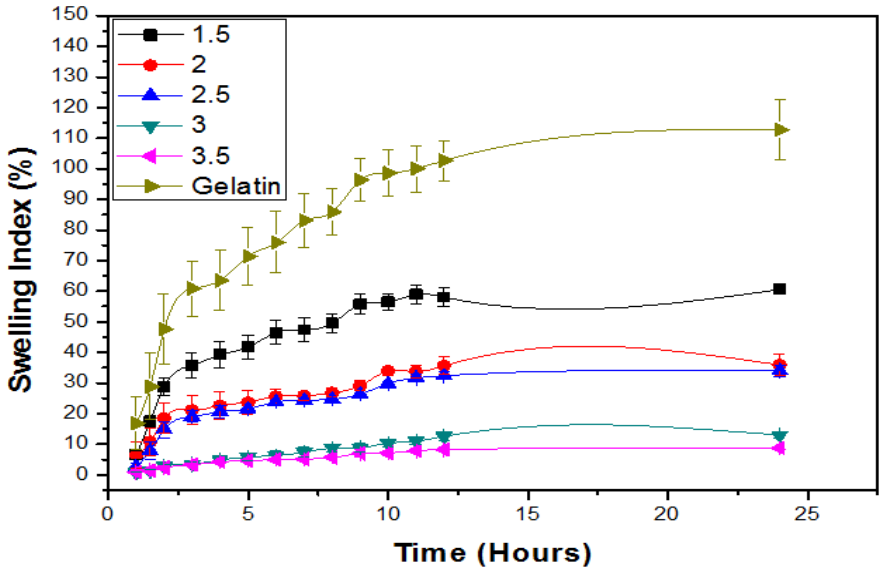


Fig 12: - Swelling index of different hydrogel formulation

The water absorption property of the gel influences not only the maintainance of the gel shape but also the cell growth. Swelling behaviour and structural stability of gel are critical to their practical use in tissue engineering [23]. Most natural polymers steadily swell in biological fluids. Swelling behaviour of the gel strongly depends on the pH value of implantation site. With increase in percentage of guargum the swelling behaviour of the gel increases. The swelling behaviour characterization is shown in the Fig**. 10.**

In vitro Drug release study of hydrogels

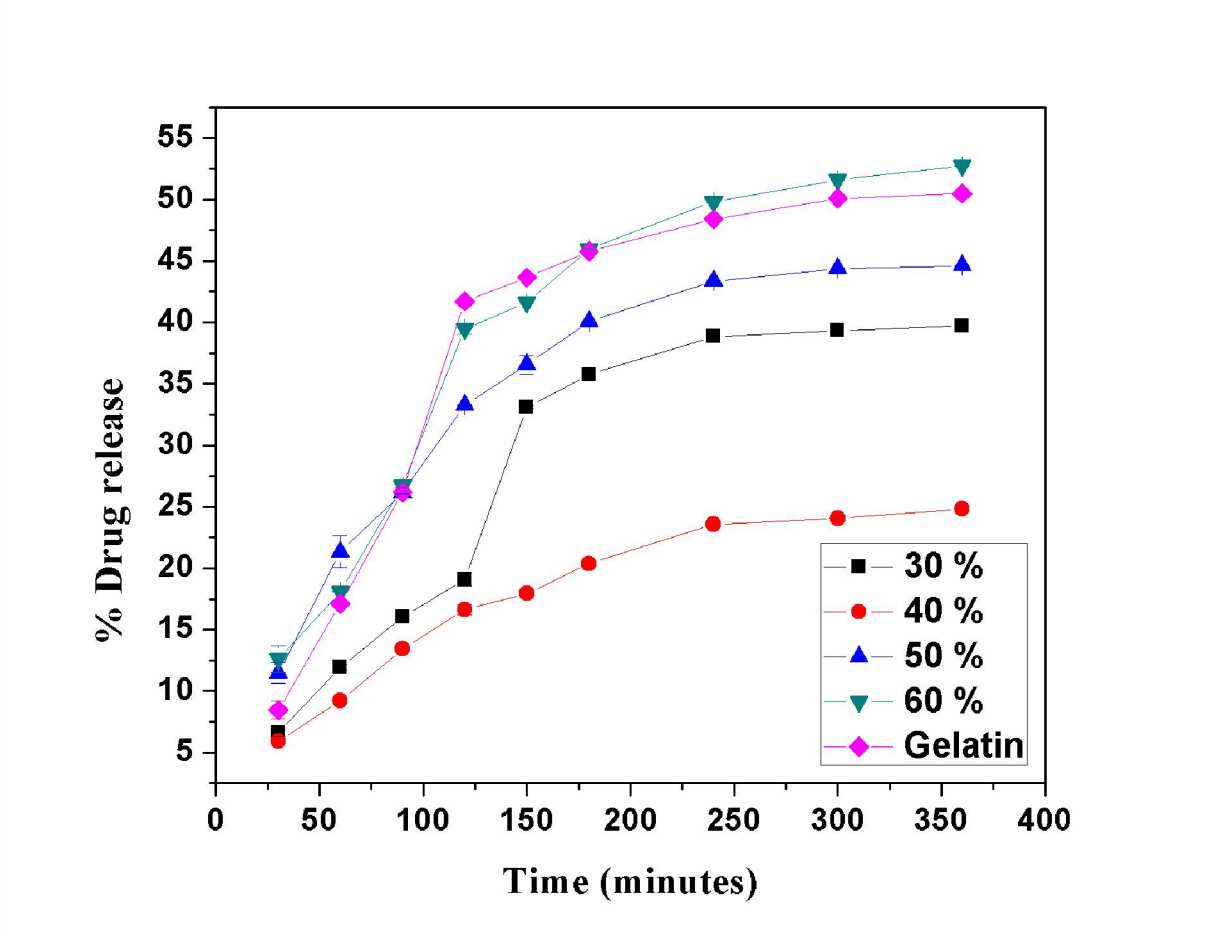


Fig 13: - In vitro drug release study of hydrogels

The drug release study of metronidazole from the hydrogels was conducted for 12 h in triplicates. The cumulative percentage of drug released from the different

compositions has been shown in **Fig. 11.** From the results we observed that the release of the drug was solely dependent on the gelators and the guargum concentrations [24]. The release rate increased with the increase in the guargum concentration and a corresponding decrease in the gelatin concentration. The GG-60 showed approximately 52.5% release of Metronidazole in 6 h. phase separated gels have shown higher release in comparison to the solid gels i.e. GG-30 & GG-40 . In 6 hour time period, GG-30, GG-40 and GG-50 showed approximately 35%, 25% and 43% release of the drug respectively. Metronidazole is slightly soluble in water. The solubility of Metronidazole dissolve was 0.333 mg/mL of gel solution. The release of the Metronidazole was higher from the composition containing higher proportions of guargum. The mechanisms that involved in the release of Metronidazole may be due to diffusion and erosion.

Cell viability assay of hydrogels

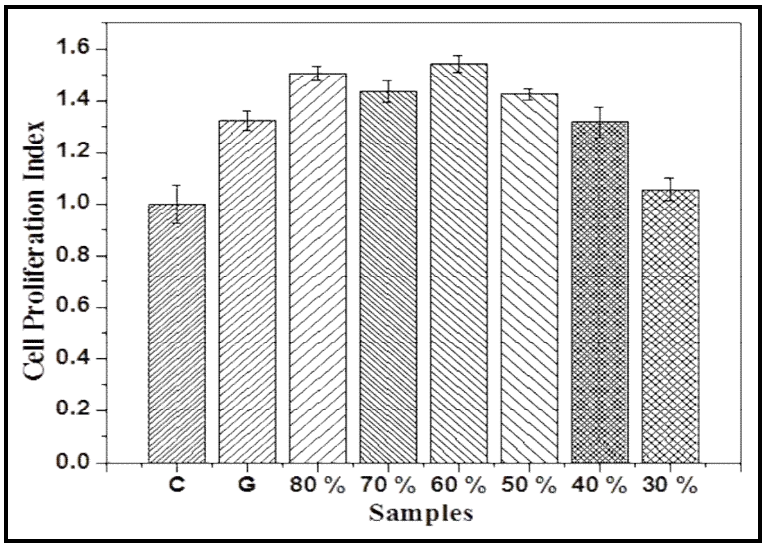


Fig 14: - MTT Assay of hydrogel formulations using MG63 cells

Biocompatibility of the samples was analyzed through MTT assay [25]. In this study, samples showed better proliferation in comparison to control (Tissue culture plate

(C)). Among the samples, 60% showed highest proliferation index of about 1.54 fold increase in comparison to control, followed by 80 % of about 1.5 fold, 70 % of about 1.43 fold, 50 % of about 1.42 fold. Samples 40% and gelatin (G) showed similar proliferation index of about 1.3 fold increase in comparison to control (c). Sample 30% showed a proliferation index same as control TCP of about 1.05. MTT assay showed that with increase in percentage of guar gum the cell proliferation index also increased **Fig. 12**. MTT results concluded that the samples prepared were biocompatible and can be used for in vivo studies.

Hemocompatibilty study of hydrogels

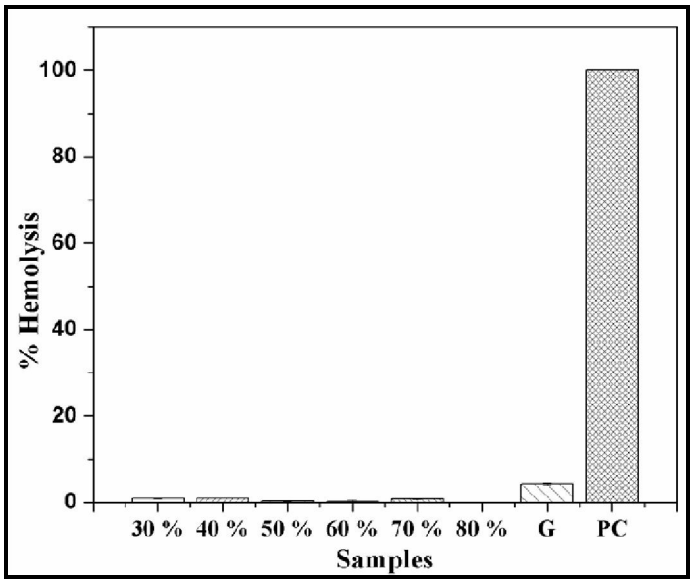


Fig 15: - Hemocompatibility study of hydrogels using human blood

Hemolysis study is generally used to assess the hemocompatibility of a biomaterial [26]. Among the samples gelatin gel showed maximum % hemolysis of about 4.3 %. The samples 40%, 30% and 70% showed same level % hemolysis of about 0.94%, 0.88% and 0.82%, respectively. Sample 50% and 60% showed % hemolysis of about 0.29% and 0.23%, respectively. Sample 80% showed the least percentage of

hemolysis and which followed same basal level as negative control (~0%) **Fig. 13**. Samples having hemolysis rate less than 5 % is highly hemocompatible, 5% -10% is hemocompatible and the samples which are having more than 20% are non-hemocompatible. Here all the samples were found to be non-hemolytic and highly hemocompatible.

CONCLUSION

In this present study, drug delivery system of genipin crosslinked phase separated guar gum and gelatin hydrogels were prepared. In phase contrast microscopy and cell kinetics study, samples containing 60% guar gum visually showed good phase separation solidifying in least amount of time (4h) . In swelling study, 60% guar gum sample showed intermediate swelling properties compared to the lower concentration of guar gum samples. In MTT assay, cell proliferation index increased as the concentration of guar gum increased and gave a maximum in the sample with 60% guar gum sample (1.54 fold increase when compared to TCP. This shows that cells got saturated in 60% guar gum samples. In hemocompatibility test, all the samples showed hemolysis of less that 5%, with 60 % sample having the second least percentage of hemolysis. This shows that the 60% sample was highly hemocompatible. This trend was also observed in drug release study in which 60% guar gum showed 52.5% (approx.) release of Metrinadazole in 6h. From these studies we can conclude that samples containing 60% guar gum was more suitable for drug delivery and tissue engineering applications.

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