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RESEARCH ARTICLE

REVIEW ON: RECENT ADVANCES IN THE STATE OF THE ART OF IN SITU FORMING INJECTABLE HYDROGEL SYSTEMS FOR THERAPEUTIC APPLICATIONS.

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Abstract

In situ injectable gelling systems have been extensively investigated with the aim of being applied for minimally invasive drug delivery or injectable tissue engineering. This article explores the injectable in-situ gelling system for prolonged release parenteral drug delivery system and their strategies of preparation. Here, we describe in situ-forming injectable hydrogel systems, prepared usinga variety of chemical cross linkers or physical interactions, for application in drug delivery. There are many newer approaches for in situ injectable hydrogels that can be delivered in minimally invasive techniques such as injection, ocular or nasal administration while protecting drugs or cells from the hostile environment. Recently, the Michael addition reaction between thiol and vinyl groups, the click reaction between bis (yne) molecules and multi arm azides, and the Schiff base reaction have been investigated for generation of injectable hydrogels, due to the high selectivity and biocompatibility of these reactions. Non-covalent physical interactions have also been proposed as cross linking mechanisms for in situ forming injectable hydrogels. Hydrophobic interactions, ionic interactions, stereo-complex formation, complementary pair formation, and host-guest interactions drive the formation of 3D polymeric networks. In particular, supramolecular hydrogels have been developed using the host-guest chemistry of cyclodextrin (CD), which allows highly selective, simple, and biocompatible cross linking. Finally, we review the current state of the art of injectable hydrogel systems for application in drug delivery, cell therapy and tissue regeneration.

Introduction:-

Hydrogels are the polymeric materials with three dimensional networks, which have gained much attention in biomedical fields as carriers for drugs, protein, cells, and others because of their good biocompatibility, solute permeability and tunable release characteristics [1].The retaining ability of a large amount of water within their structures which results in high water content and soft-surface properties is the character that makes them compromised on the surrounding tissues and leads to a good biocompatibility. Since the development of hydrogels in 1960s, numerous studies on adapting hydrogels as biomaterials have been reported. Especially, the in situ forming hydrogels which usually show sol-to-gel transition at the in-situ site where they are administrated into the body,

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exhibit promising potentials for clinic applications. It is more practicable to apply in-situ forming hydrogels to tropical drug delivery, injectable implant, tissue engineering scaffold and so on [2-4]. The drug/cell can be mixed with the aqueous sol for convenient administration. The development of injectable in situ forming drug delivery systems has received a considerable interest over the last decade. In situ gelling systems could potentially alleviate several drawbacks associated with contemporary regenerative medicine approaches and scaffolds. Primarily, they minimize the invasiveness of the open surgical technique and can conform to complex 3D geometries, which is critical in implant drug delivery system, repair of trauma, and regeneration post-tumor resection. More importantly, this allows for delivery of cells and growth factors locally, which could potentially lead to faster and complete regeneration [5].

Injectable hydrogels which can form a gel state after injection into the body are advantageous in biomedical applications due to their high shape ability which can fill irregular defect sites [6], and minimally invasive delivery of a large amount of hydrogel together with bioactive molecules/cells [7] that can eliminate large incision and reduce recovery time, risk of infection, and pain to the patient [8]. Injectable polymers have drawn considerable attention as promising biomaterial for drug delivery and regenerative medicine. Multiple biocompatible and biodegradable polymers are routinely employed as carriers of injectable DDS in order to diminish the drug side-effect, especially for local administration and delivery when used for anticancer chemotherapy.

Many polymeric materials for injectable DDS, such as nanoparticle [9-11], microsphere [12, 13], polymeric micelles [14-15], liposomes [16-18], and hydrogel system [19-20], have been investigated and developed. Although some formations of them have succeeded in clinical applications there still remain many problems that need to be addressed. One of the recent manifestations of stimuli responsive polymers lies in in-situ forming DDS by sol-gel transition for in-situ forming hydrogel because it is feasible to use them as carrier for local administration [21]. As representatives of stimuli-responsive polymer for in-situ forming hydrogel, there are several candidates that include thermoresponsive polymers such as N-isopropyl acrylamide copolymer [22], polyethylene glycol–polypropylene glycol–polyethylene glycol (PEG–PPG–PEG) triblock copolymer [23], and polyethylene glycol–poly L-lactic acid–polyethylene glycol (PEG–PLLA–PEG) triblock copolymer [24]. These thermoresponsive polymers exhibit thermo-dependent sol–gel transition in aqueous solution via hydrophobic interaction. The advantage of these kinds of polymers is in their ability to avoid toxic cross-linkers which are usually employed to form hydrogel.

However, local injection of thermoresponsive polymers is operationally difficult. Their thermoresponsiveness is too sensitive for injection by syringe pump and for this reason they must be cooled down to below the transition temperature before they can be injected. Furthermore, ion-mediated cross-linked hydrogels, such as alginates, which form a gel upon contact with divalent cations, have been widely researched as injectable in-situ forming DDS and tissue engineering because of their biocompatibility [25, 26]. Many alginate derivatives such as lectin-modified alginate [27] and RGD containing alginate [28] have also been synthesized. Despite many of their applications, alginate hydrogels have limited use because of their low shelf lives.

In- situ gels are smart polymeric systems which are capable of undergoing rapid sol-to-gel transformation triggered by external stimulus such as temperature modulation, pH change or ionic exchange etc. on instillation from which the drug gets released in a controlled and sustained manner to obtain defined blood levels over a specified time, as well as by avoiding the systemic circulation it reduces toxicity in normal tissues [29, 30]. These are liquid at room temperature but undergo gelation when there is environmental changes like change in pH or temperature, ionic concentration, osmolarity or irradiation[31], magnetic field, ultrasound or visible wavelength in case of photosensitive systems in responsive systems [32, 33].

In contrast to very strong gels, they can be easily applied in liquid form to the site of drug absorption. At the site of drug absorption they swell to form a strong gel that is capable of prolonging the residence time of the active substance. Mostly, biodegradable polymeric materials such as natural polymers including polysaccharides and polypeptides, and synthetic polymers such as PLA and PLGA are used for its formulation [34].

A key requirement of in-situ depot-forming systems for local delivery is the injectability using standard gauge needles either in a vial/syringe or a pre-filled syringe configuration [35]. Ideally, an in - situ gelling system should be a low viscous, free flowing liquid to allow for reproducible administration. In-situ gels are administered by injectable as well as oral, ocular, rectal, vaginal and intra peritoneal routes. [36].

Need of In-situ gelling system:-

In recent years, the development of in situ gel systems has received a considerable attention as polymeric drug delivery systems. The importance of in situ forming matrix systems is related to several advantages such as, for instance, easy application, use of non-toxic carriers, simple and economical elaboration, prolonged residence time and controlled drug release. Moreover these systems avoid painful surgical procedures to insert solid implants [37]. The in situ forming gel systems are designed such that they are fluid prior to injection. Once injected, the formulation responds to a change in the environment to give a high viscosity or solid depot at the injection site. The most studied thermo sensitive polymers are the pluronics which are poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) block copolymers. Those polymers exist as a mobile viscous liquid at reduced temperatures but form a rigid semisolid gel network with an increase in temperature. Unfortunately, pluronic gels are obtained at high polymer concentrations only (between 20 and 30%) and have been shown to erode rapidly [38]. Traditional drug delivery system has got many disadvantages such as high plasma concentration in the systemic circulation when the drug is administered parenterally that result in undesirable side effects [39, 40]. Additionally, many drugs undergo high first pass metabolism and therefore show less bioavailability when given orally. *In situ* gel formulations offer an interesting alternative for achieving systemic drug effects of parenteral routes, which can be inconvenient for oral route, which can result in unacceptably low bioavailability and passes the hepatic first-pass metabolism, in particular of proteins and peptides[41]. Although, parenteral route offers rapid onset of action with rapid declines of systemic drug level, it requires frequent administration which ultimately leads to patient discomfort.Novel technologies have been developed to overcome these problems by reducing total no of injection throughout the effective treatment. In-situ drug delivery system is one of the most effective controlled release drug delivery systems that have been formulated using injectable and biocompatible smart polymer. This system offers attractive opportunities and provides sustained release of drug with less pain and less invasive technique thereby reducing frequency of administration and improving patient compliance.

Advantage:-

1. In–situ gel offers sustained and prolonged action in comparison to conventional drug delivery
2. Production of such device is simple and thus minimizes manufacturing costs and associated investments.
3. Ease of administration and improved patient compliance.
4. Deliverance of accurate dose
5. *In situ* gels can also be engineered to exhibit bioadhesiveness to facilitate drug targeting, especially through mucus membranes, for non-invasive drug administration.
6. *In situ* gels offer an important "stealth" characteristic *in vivo*, owing to their hydrophilicity which increases the in vivo circulation time of the delivery device by evading the host immune response and decreasing phagocytic activities.
7. Reduced toxic and side effects as the drug is delivered locally at the site of action therefore no harms to the healthy tissues can be seen.

Disadvantage:-

1. It requires high level of fluids.
2. It leads to degradation due to storage problems.

Approaches In The Design Of In Situ-Forming Injectable Hydrogels:-

Injectable hydrogels prepared by chemical cross linking:-

Michael addition for formation of injectable hydrogels:-

Michael addition is the nucleophilic addition of a carbanion or a nucleophile, such as thiols and amines, to an α, β unsaturated carbonyl compound [42]. The reaction is highly selective under physiological conditions, without involving toxic reagents and side products. Accordingly, this reaction has been widely exploited for the preparation of injectable hydrogels for biomedical applications. For example, the Michael addition reaction can occur between thiol and vinyl sulfone (VS) or aminoethylmethacrylate (AEMA). As a polymer backbone, synthetic and natural polymers have been used for the preparation of hydrogels, such as poly (ethylene glycol) (PEG), collagen, HA, and heparin [43-47]. Cells and biopharmaceuticals can be encapsulated within such a hydrogel by simple mixing with the polymer precursor solutions. Extracel^{TM} is one of the typical injectable hydrogels created by Michael addition between a thiol modified carboxymethyl HA and gelatin modified with diacrylated PEG [43, 48-50].

Click reaction for formation of injectable hydrogels:-

Click chemistry is a Cu (I)-catalyzed reaction between azide and terminal acetylene groups, forming 1, 2, 3-triazoles [51, 52]. This reaction is widely employed for biomedical applications, because of the high yield, region specificity, absence of toxic byproducts, and rapid reactivity under physiological conditions. Using click chemistry, various hydrogels have been developed for drug delivery and tissue engineering applications [53-56]. A polymeric 3D network has been fabricated using a dipolar cyclo addition reaction between the two types of derivatives in the presence of a catalytic amount of Cu (I), at room temperature. Recently, copper-free click chemistry has also been developed using azide-alkyne cyclo addition between difluorinated cyclo octyne (DIFO3) and azide, and applied to in situ hydrogel formation [57, 60]. Simple mixing of the polymer precursor solutions with a cell suspension resulted in hydrogel formation via the highly specific click reaction of azide with acetylene, encapsulating the cells. In addition, post-modification of the hydrogel on demand, can be performed by subsequent functionalization of the remaining azide or acetylene group.

Schiff base reaction for formation of injectable hydrogels:-

Injectable hydrogels can be prepared by a Schiff base reaction between an amine group and an aldehyde group, without additional chemical cross linking reagents. The residual functional groups within the hydrogel can be used for covalent conjugation of therapeutic molecules or additional cross linking. HA, chitosan, dextran, chondroitin sulfate, and poly (vinyl alcohol) have been used for the preparation of hydrogels via the Schiff base reaction [59-61]. The gelation time and physical properties of these hydrogels are dependent on the ratio of the amine and aldehyde groups. Although the cells entrapped in these hydrogels have been reported to maintain their normal morphology, aldehyde groups can react with other amine groups in biomolecules of cells in the body during the cross linking reaction.

Enzyme-mediated injectable hydrogels:-

Tyramine-conjugated polymers have been used for in situ hydrogel formation in the presence of $H_2O_2$and horseradish peroxidase [62-65]. The enzymatically crosslinked hydrogels can be prepared within10 min, depending on the polymer concentration and the enzyme/tyramine ratio. Polymer-tyramine hydrogels with high elasticity have been used as drug delivery depot systems [63] and tissue engineering scaffolds [61-65].

Photo-cross linked injectable hydrogels:-

Methacrylated polymers have been used for in situ hydrogel formation by photo-crosslinking with a photo initiator [66]. The hydrogel precursor solution is injected into the body and is then exposed to visible or ultraviolet (UV) light. Photo-crosslinking has also been used to improve the mechanical properties and stability of physically crosslinked hydrogels [67, 68]. For example, methacrylic acid was introduced into thermosensitive polymers or electrostatically crosslinked hydrogels. The thermosensitive photopolymerized hydrogels demonstrated improved mechanical properties. However, the practical applications of photo-cross linked hydrogels are limited due to the possible toxicity of photoinitiators, the long exposure time, and the short penetration depth of light sources [69, 70].

Injectable hydrogels prepared by electrostatic interaction:-

Alginate-based injectable hydrogels:-

Alginate is an anionic polysaccharide derived from sea algae. Alginate hydrogels are formed by simple mixing of alginate solutions with divalent cations, such as $Ca^{2+,}$ Mg^{2+} and Ba^{2+}. Alginate based hydrogels have been widely used for drug delivery [71], cell therapy [72, 73], and tissue engineering [62-65]. They have been used in clinical trials and are a component of FDA approved medical products. The formation and the mechanical strength of alginate-based hydrogels can be controlled by changing the concentration and the type of cation added. For example, the rate of alginate hydrogel formation increased with increasing total calcium content in the case of $CaCO_3$/d-glucono-δ-lactone (GDL) and $CaSO_4$/$CaCO_3$/GDL systems [74]. The mechanical properties of the alginate hydrogels were improved with increasing alginate concentration, total calcium content, molecular weight, and glucuronic acid content of the alginate. However, alginate hydrogels formed by ionic interaction are not stable in the body, because ionic molecules diffuse out from the hydrogels into the body fluid [73-75]. In addition, the formation of alginate hydrogel is difficult to control, and the hydrogel has poor cell adhesion [76]. To improve the stability and mechanical properties of this type of hydrogel, highly stretchable and tough alginate hydrogels have been prepared by additional covalent cross linking [77, 78].

Chitosan-based injectable hydrogels:-

Chitosan is an alternating copolymer of 2-acetamido-2-deoxy-β-d-glucopyranose and 2-amino-2-deoxy-d-glucopyranose derived from naturally occurring chitin. Chitosan can form a hydrogel complex with polyanionic molecules via electrostatic interaction [79].Temperature and pH-responsive chitosan-based hydrogels have been prepared with polyol-salts possessing a single anionic head, such as glycerol-, sorbitol-, fructose-, or glucose-phosphate salts (polyol- or sugar-phosphate) [80-83]. The driving force behind hydrogel formation includes hydrogen bonding, electrostatic interaction, and hydrophobic interaction between chitosan and polyol-phosphate salts. The chitosan solution remains liquid at physiological pH and turns into a hydrogel at body temperature. Drugs and cells can be easily entrapped within the hydrogel by mixing them with the precursor solution at low temperature prior to injection.

Stimuli-responsive injectable hydrogels:-

Temperature-responsive injectable hydrogels:-

Some polymers undergo solubility changes and phase transitions in response to environmental temperature [84, 85]. This threshold is referred to as the lower critical solution temperature (LCST) [86]. For example, poly (N-isopropylacrylamide) (PNIPAAm) undergoes phase transition at temperatures above 32°C in an aqueous solution. Because the LCST of PNIPAAm is increased by copolymerization with a hydrophilic polymer, in situ gelation temperature can be adjusted to body temperature [84]. In addition, some amphiphilic polymers were used for hydrogel formation, by micellar packing, in response to temperature changes [87]. Linear and star shaped block copolymers composed of central hydrophilic polyethylene oxide (PEO) and terminal PNIPAAm, showed a temperature-responsive behavior, forming relatively strong injectable hydrogels [86]. Recently, biodegradable temperature-responsive hydrogels were developed for biomedical applications by combining non-biodegradable PNIPAAm and biodegradable polymers [88-90]. The HA-g-PNIPAAm conjugate forms a hydrogel network, exhibiting reversible temperature-responsive solubility [89]. The degradation rate, swelling ratio, and cytocompatibility of the hydrogels can be controlled by changing the weight ratio of PNIPAAm to HA for tissue engineering applications. A PEO-PPO-PEO triblock copolymer, under the tradename of Pluronic®, is one of the most commonly used thermosensitive hydrogels for biomedical applications. Dehydration and increasing hydrophobicity of the PPO block with increasing temperature results in micelle formation, which is the driving force for in situ hydro-gel formation. This hydrogel formation is dependent on the concentration and temperature of the polymer precursor solution. Pluronic® with a different composition and molecular weight of copolymer has been used for applications in drug delivery, gene delivery, tissue adhesion prevention, and tissue engineering [91]. However, Pluronic® systems have the disadvantages of having weak mechanical strength and being non-biodegradable. Biodegradable PEG-PLGA-PEG also forms a thermoresponsive hydrogel similar to the PEO-PPO-PEO triblockcopolymer systems [92]. Despite the wide clinical exploitation of PLGA based copolymers with FDA approval, they are known to cause harmful side effects to biomolecules, cells, and tissues in some cases after they are degraded to acidic monomers [93, 94]. To overcome these issues, porous devices, microparticles, and hydrogels have been developed using this type of polymers [94].

Dual-responsive injectable hydrogels:-

The main disadvantage of physically cross linked thermo sensitive hydrogels is their weak stability and mechanical properties in the body. Accordingly, dual-responsive hydrogels have been developed to alleviate these problems. For example, temperature and pH-responsive hydrogels have been developed using PNIPAAm-based copolymers. PNIPAAm is copolymerized with pH-responsive segments, such as poly (propylacrylic acid) (PPAA), poly (N-isopropylmaleamic acid) (PNIPMAA), and poly (methacrylic acid) (PMAA) [95]. These synthetic polymers are not only temperature responsive but also significantly pH responsive due to the presence of carboxyl groups. In addition, temperature and pH-responsive hydrogels have been prepared with multiblock copolymers [96]. The pH-responsive sulfamethazine oligomers (SMO) have been conjugated to both ends of thermoresponsive poly (ε-caprolactone-co-lactide)-PEG-poly (ε-caprolactone-co-lactide). The resulting SMO–PCLA–PEG–PCLA–SMO multiblock copolymer solution shows a reversible sol-gel transition at pH 7.2 and body temperature. The mechanism of hydrogel formation is the hydrophobic interaction between SMO and PCLA blocks. These temperature and pH (dual) responsive hydrogels have enhanced mechanical strength and prevent gelation of the precursor solution in the needle during injection into the body. Other dual-responsive hydrogel systems that enhance the mechanical properties of physically cross linked hydrogels have also been developed using photo and temperature responsive hydrogel systems [97, 98].

Supramolecular injectable hydrogels prepared by self-assembly:-

Self-assembling injectable hydrogels by complementary binding:-

Self-assembling hydrogels have been developed using various complementary bindings, such as ligand-receptor pairs [99-101], antigen-antibody pairs [102-104], and base-pairing interactions [105-107]. Because ligand-receptor pairs have an extremely high binding affinity, the formation of a complex between a receptor and a ligand can be used to drive formation of injectable hydrogels. For example, a streptavidin-biotin pair has been used for the preparation of injectable hydrogels. PLA-PEG–biotin microparticles have been cross linked with avidin to generate 3D porous matrices that self-assemble at the injection site. In addition, multiple repeats of tryptophan rich domains and proline rich peptide domains have been used for hydrogel formation [102]. The amount of crosslinking protein affected the hydrogel formation rate, as well as the physical strength of the hydrogels. Moreover, growth factors could be added to the hydrogel precursor solution to promote cellular functions within the hydrogel. Antibody-antigen interaction has also been used for the formation of an injectable 3D network [104-106]. Simple mixing of antibody-conjugated polymer and antigen-grafted polymer solutions can result in hydrogel formation. Treatment with free antigen or free antibody also affects the physical properties of these hydrogels. In addition, self-assembling hydrogels have been prepared using three complementary branched DNA sequences [105-107]. However, the relative difficulties in mass production and chemical modification of biomolecules, as well as the potential safety issues involved, should be addressed to expand the applications of these hydrogels to therapeutic purposes.

Self-assembling injectable hydrogels by host-guest interaction of cyclodextrin:-

As an alternative to biological complementary binding pairs, self-assembling hydrogels have been developed using host-guest interaction of the cyclodextrin (CD) family. CDs are series of natural cyclic oligosaccharides composed of six, seven, or eight d-glucopyranoside units (α, β, and γ-CD). They have a hydrophobic inner cavity, by which they can generate an inclusion complex with other guest molecules, such as PEG, adamantane, and cholesterol [108]. Recently, injectable hydrogels that make use of an inclusion complex of CD have emerged as another series of promising physical hydrogels that can be used in various biomedical applications [109-111]. PEG can penetrate the inner cavity of α-CD to generate an inclusion complex. Injectable hydrogels have been created by mixing high molecular weight PEG and α-CD in aqueous solution [109]. This type of hydrogel is reversibly thixotropic and non-degradable high molecular weight PEG is not ideal for in vivo applications. To improve the stability of the hydrogel, PEO-PPO-PEO was used to make a complex with α-CD. β-CD and adamantane, and β-CD and cholesterol pairs have also been investigated for the preparation of injectable hydrogels [110-112]. However, CD-based hydrogels have an intrinsic limitation in in-vivo applications, due to the low binding affinity of CD to guest molecules and the low stability of the resulting hydrogels in the body [112].

Evaluation And Characterization Parameters For Injectable In-Situ Gels:-

Clarity:-

The clarity of formulated solutions determined by visual inspection under black and white background [32, 113]

Texture analysis:-

The consistency, firmness &cohesiveness of in situ gel are assessed by using texture profile analyzer which mainly indicated gel strength & easiness in administration in vivo higher value of adhesiveness of gel are needed to maintain an intimate contact with mucus surface [32, 113].

pH of gel:-

The pH can be determined using pH meter. The formulation is taken in beaker & 1ml NaOH added drop wise with continuous stirring and pH is checked by using pH meter [114].

Inner morphology of the dual cross linked hydrogels:-

The inner morphology of the dual cross linked hydrogels are investigated using scanning electron microscope. Briefly, the lyophilized hydrogel samples are surface gold sprayed and observed using SEM at an accelerating voltage of 10 kV. At last, average pore diameter and pore area are quantified using Image software [115].

Gel-Strength:-

This parameter can be evaluated using a rheometer. Depending on the mechanism of the gelling of gelling agent used, a specified amount of gel is prepared in a beaker, from the sol form. This gel containing beaker is raised at a certain rate, so pushing a probe slowly through the gel. The changes in the load on the probe can be measured as a function of depth of immersion of the probe below the gel surface [116].

Gelling capacity:-
In-situ gel is mixed with tissue fluid in the specific proportion to find out gelling capacity of injectable product. The gelation assessed visually by noting the time for & time taken for dissolution of the formed gel [117].

Gelation temperature and in situ gel formation studies:-
One millilitre of the polymeric system is transferred in a small bottle; 1ml deionized water is added and incubated at 37.4 ºC. Separately 1 ml of the polymeric system is introduced to dialysis against PBS pH 7.4 in cellulose tube at 37.4 ºC for 120 min. The system is monitored every 15 min to see if gelation has occurred. For the in vivo evaluation of the system, 0.1-0.3 ml of the polymeric composition is injected subcutaneously into the back of the neck or abdominal area of the rats. After 120 min the marked area is opened through a surgery operation and the fate of the hydrogels are evaluated [118].

Rheological studies:-
The viscosity measured by using Brookfield viscometer, cone & plate viscometer [119].

Water content and swelling studies:-
The prepared dual crosslinked hydrogels are transferred to beakers that containing 37 ºC distilled water to investigate their water content. The swollen hydrogels are taken out and surface water blotted at determined time point to weigh their mass until reaching a constant mass. The swollen hydrogels are lyophilized and the dry gel is also weighed. Equilibrium water content (EWC) and swelling ratio are determined by the following equations [120, 121]:

$EWC\ (\%) = W_e\text{-}W_d/W_e \times 100$ ……..(1)
$Swelling\ Ratio\ (\%) = W_S\text{-}W_d/W_d \times 100$……..(2)

In equation (1) W_e is weight at equilibrium swollen state and W_d is dry weight. In equation (2) W_s is the swollen weight at given time and W_d is dry weight.

In vitro degradation studies:-
Degradation of hydrogels is assessed at 37ºC with continuous shaking in a shaking water bath and simulated body fluid (SBF) is changed every two days. At predetermined time intervals (1-6 weeks), hydrogels are rinsed with SBF and lyophilized. The vacuum dried hydrogels are weighed and weight loss percentage is calculated by the formula [122]:
$Weight\ Loss\ (\%) = W_i\text{-}W_d/\ W_i \times 100$
Here W_i is initial weight of the sample, W_d is dry weight of the sample.

In-Vitro Drug Release Studies:-
The drug release studies are carried out by using the plastic dialysis cell. The cell is made up of two half cells, donor compartment and a receptor compartment. Both half cells are separated with the help of cellulose membrane. The sol form of the formulation is placed in the donor compartment. The assembled cell is then shaken horizontally in an incubator. The total volume of the receptor solution can be removed at intervals and replaced with the fresh media. This receptor solution is analyzed for the drug release using analytical technique [123, 124]

Histopathological studies:-
For the histological studies, the animals are sacrificed and the intact shaved skin of injection area and the left and right testicles are isolated and washed with normal saline to remove blood and are fixed for 24 h at 4 ºC, and then dehydrated with a 50-100% v/v ethanol series with a final change in xylene, before embedding in paraffin. Five micrometers sections are cut and mounted onto positively charged slides, which is heated at 55 ºC to ensure adherence of the sections. For staining, sections are dewaxed in xylene, then rehydrated in a 100-50% ethanol series, and quickly rinsed in distilled water. The sections are observed under high magnification (_100/x400) light microscope to check histopathological changes [125, 126].

Sterility testing:-
Sterility testing is carried out as per the IP 1996. The formulation is incubating for not less than 14 days at 30-35ºC in the fluid thioglycolate medium to find the growth of bacteria & at 20-25ºC in Soya bean casein digest medium to find the growth of fungi in formulation [127].

Accelerated stability Studies:-
Formulations are placed in ambient colour vials and sealed with aluminium foil for a short term accelerated stability study at 40±2°C and 75±5% RH as per International Conference on Harmonization (ICH) states Guidelines. Samples are analyzed every month for clarity, pH, gelling capacity, drug content, rheological evaluation, and in vitro dissolution [128]

In vivo studies:-
The experiment is performed in accordance to the guidelines of international animal studies approved by the ethics committee. Male rats 8 weeks old, 250-300 gram weight that are given food and water ad libitum are used. During the whole study, uniform feed and free water is supplied. In this study the serum testosterone level of four groups (A, B, C, and D) of male rats (n = 6) at each time intervals after treatment is compared with the initial base line of the same group. Group A received no injection to verify the natural fluctuation of serum testosterone concentration during the study. Group B received µl injection of drug in pure form. Group C received µl injection of the in situ gel forming system containing drug. Group D received 100 µl injection of the drug free in situ gel forming system as placebo. All samples are injected subcutaneously at the back of the neck of the rats. The level of serum testosterone is determined for 3 days as testosterone base line in each group before starting the study. Blood samples are collected at 3 and 6 h in the first day and on days 2, 3, 7, 14, 21, 28, 35, 42 directly from the heart of the animals. The blood samples are centrifuged for 15 min at 14,000 rpm to separate serum. The serum testosterone level is determined by Liaison testosterone kit as direct chemiluminescence immunoassay [129, 130]. Results are expressed as mean ± standard error (SE), or mean ± standard deviation (SD), and significance between two groups is determined by Student t-test.

Conclusion:-
In this review, we have summarized recent progress in the art of design of in situ injectable gelling systems. The gelling process should occur under mild conditions for biomedical applications without damaging incorporated pharmaceuticals and cells. Therefore, cross links have been prepared in aqueous systems by benign chemical reactions such as redox/photo-polymerization, Michael addition, click reactions, enzymatic reactions; or physicochemical association of the molecules including thermo gelation, ion-induced gelation, inclusion complex formation, stereo complex formation, and complementary binding processes. Compared to the conventional therapy, the injectable hydrogel systems provide some advantages, such as reduced toxicity in normal tissues, localized and sustained delivery of the drugs in the tumor vicinity, more efficient cell apoptosis, as well as tumor growth inhibition. Injectable hydrogels prepared by chemical cross linking demonstrate good mechanical properties, but in vivo applications have been limited due to the possible cytotoxicity of the reactive chemical cross linkers. In contrast, injectable hydrogels prepared by physical cross linking can be formed easily without reactive chemical reagents, but the hydrogels have poor stability and mechanical properties in the body. Supramolecular injectable hydrogels are fabricated by self-assembly of receptor-ligand pairs, complementary pairs, and host-guest pairs.

Future Scope:-
This smart polymeric injectable gelling system has come out as a promising drug delivery system mainly for the very potent drugs like anticancer molecules and protein and peptide drugs. The manufacturing process is very simple which lessen the cost of the product which are otherwise very expensive. Also they possess the tremendous capability to deliver the drug effectively to the site with minimal or no systemic side effects. It can be formulated into a suitable formulation for easy injection prior to in-situ gelation and show controlled release. Therefore it can be used for effective delivery of anti cancer molecules to the site with no or very less systemic bioavailability and no harm to the healthy organs. Future use of biodegradable and water soluble polymers for the in-situ gel formulations can make them more acceptable and excellent drug delivery systems for use of injectable drug depots for systemically active compounds. The extensive work demonstrating the use of these materials for delivery of insulin, a signaling hormone that acts on systemic sites in the liver and skeletal muscle, points to an obvious application for skin-associated delivery of depots containing systemically active drugs. In particular, there is logical application for these technologies in the delivery of biologics, which remain very difficult to administer orally, in order to enable therapeutic use by patients at home that would otherwise require infusion in a clinic. Another application for injectable biomaterials that aligns with standard practice is in vaccination. Hormonal therapy has been the main treatment of advanced and metastatic prostate and breast cancers. Initially luteinizing hormone-releasing hormone (LHRH) agonist therapy consisted of daily subcutaneous injections of LHRH agonists (e.g., leuprolide, goserelin, triptorelin) are successful line of therapy.

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