A peel-able onion inspired multi-membrane chitosan hydrogel with reversible sol-gel transition, on-demanding dissolution for intestine-selective controlled release, shape memory, 3D printed E-skin biosensor and microchannel system

By

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Abstract

There are numerous properties of biomaterials, existing in the nature which are still unknown to the scientific community. Different modifications to the biomaterials can unlock new properties of these materials. This leads to the development of whole new type of material which can be used as an alternative to the existing materials and contribute to wide range of new applications. Stimuli-responsive hydrogels are one of the best strategies for controlled drug delivery, tissue engineering and biosensing based applications. Here in we report, a novel pH-sensitive and biodegradable hydrogel systems based on the carboxymethyl chitosan (CMC). The mechanical property of CMC changes from acidic to basic medium. This property is the reversible sol-gel conversion (solution phase to gel phase) of CMC solution based on the pH changes, and it was used in this work to synthesize peel-able onion like multimembrane hydrogels for a controlled fluorescein drug delivery model to intestine in gastro-intestine systems. Dissolution rates of the hydrogels was altered by using different concentrations of CMC and different number of hydrogels layers. Furthermore, CMC was used as a novel 3D printable bioink for potential biomaterial-based applications. The resulting CMC 3D printed structures were further used as a real-time wireless biosensor and for designing microfluidic channels. The mechanical properties and characterization of the CMC were studied by using rheology tests, FT-IR and ^{1}H NMR.

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List of Abbreviations

CMC – Carboxymethyl chitosan

PVA – Polyvinyl alcohol

HCl – Hydrochloric acid

PDMS – Polydimethylsiloxane

NaOH – Sodium hydroxide

MWCNT – Multi-wall carbon nanotubes

Chapter 1: Introduction

1.1 Background

Recent growth and revolution in materials research has led to the discovery of various stimuli responsive smart materials, which are the class of materials with ability to respond to external stimulus. The response can be in the terms of change in shape, physical properties or chemical structure on application of external stimulus, such as pH, temperature, optical radiations, ionic strength, etc.[1-4]. Smart materials based on the biomaterials are of great interest because of their biocompatibility, non-toxicity characteristics and their abundance availability in nature. These hydrogels are widely used in biomedical applications like drug and gene delivery systems, tissue engineering, biosensors, artificial muscles, etc.[5-14]

Chitin is the second most abundant natural polymer on the planet after cellulose and is biosynthesized by a large group of living organisms[15, 16]. Its function in nature is mostly to impart reinforcement and strength to the natural structures. Chitosan is a derivative obtained from chitin by partial deacetylation under alkaline or enzymatic hydrolysis[17]. As a polysaccharide, chitosan have been wildly used in biomedical applications due to their biocompatibility and biodegradability[18]. However, chitosan with pKa value of around 5.6 - 6 can only be dissolved in an acid solution, thus limiting its applications at physiological conditions[19-21]. Synthesis of water soluble chitosan made by carboxylation of the chitosan enhances and imparts many new properties to the material such as moisture retention, gel-forming capability, and antibacterial activity[22-24]. Introduction of these properties expand their application areas such as absorbing

metal ions, antimicrobial materials, cell therapy, tissue engineering, biosensors and drug delivery[15, 25-28]. Depending on the site of substitution, it can either be an O-carboxymethyl chitosan with positions of the D-glucosamine unit at the C-3 and C-6 or an N-carboxymethyl on the -NH_2 group[29]. Chitosan based hydrogels have been extensively studied for their applications in wound healing, scaffold preparation in tissue engineering, as drug delivery materials [27-31].

Onion inspired layer by layer structure provides a versatile method for controlled delivery with spatial control of structure[32, 33]. Multi-membrane hydrogels are prepared by layer by layer synthesis of hydrogels one after another with intermembrane spaces between each layer. Ladet et al.[32] reported a novel method for preparing the chitosan based multi-membrane hydrogel systems with the spaces between the membranes using multi-step interrupted gelation process which was carried out in a controlled physico-chemical condition to synthesize the hydrogel. In this, the variations of balance between the hydrophobic and hydrophilic interactions was the most important thing and was responsible for inducing shrinking of the neutralized hydrogel. Interrupting the neutralization in 1M NaOH, lead to the formation of spaces between the neutralization hydrogel and in this manner, hydrogel layers were deposited on the core. However, synthesis of multi membrane hydrogels which can be further peeled off into single layered gel membrane- a true onion like system is still challenging along with the time required for the synthesis of these hydrogels.

There has been recent interest in developing the hydrogels which can be dissolved on demand. The importance of dissolution of hydrogels is desired for removal of hydrogel after it has achieved its assigned function and also for the site-specific drug delivery systems[34]. Recently, control of dissolution time over the hydrogel have been synthetically governed by adding chemical moieties in the hydrogels which can be cleaved by enzymatic degradation or ester hydrolysis[35, 36].

Different reactions such as thiol−thioester exchange[37], thiol−disulfide exchange[38], retro-Michael-type addition[39], and retro-Diels−Alder reactions[40] have been determined to add tailorable degradation rates to the hydrogel. Current research in the dissolution of hydrogels still needs a lot of advancements to develop a hydrogel which can be dissolved into nontoxic residues and provide a control over the degradation rate.

In this thesis, we report a pH sensitive CMC which exhibits a fast sol-gel conversion. Dissolution of hydrogels in the wide range of basic concentrations proves to be a vital solution to the development of dissolvable hydrogels. This reversible sol-gel conversion property was further exploited to make peel-able onion inspired hydrogels, which were used for controlled drug delivery in gastric-intestine environment. Further extending the applications of CMC, it was used in origami based shape memory materials, 3D printable bioink for microfluidics, and 3D printed biosensors. CMC solution was used to cover one side of filter paper to design paper-origamis with potential pH sensitive shape memory applications. CMC was also used as a novel 3D printing bioink for printing hydrogel materials with potential applications in designing microfluidic channels in polymers and for making a CMC hydrogel based e-skin sensors with multifunctional sensing applications. Different shapes of microfluidic channels were made by altering the shapes of CMC 3D printed hydrogels, which was used as a template to design channels in the polymers. Solution of CMC-CNT was used to make a e-skin based multifactional biosensor for sensing basic human functions like speech, motion and breathing.

1.2 Problem Definition

The need and desire to engineer new materials for biomedical applications was the main driving force behind this thesis. Biomaterials are widely being investigated for their constructive properties because of the abundance availability in nature and biocompatible characteristics. Biomaterials such as cellulose, chitosan, alginate, etc. are one of the few examples of this fruitful research. Chitosan which is derived from the chitin is the second most abundant polymer present on the planet earth after cellulose. Chitosan based hydrogels have found potential applications in the field of biomedical sciences because of their good biocompatibility and biodegradability properties. But, there are still some properties of the existing biomaterials which are unknown to the research world and can be triggered by certain chemical or physical modifications to the material structure.

Secondly, search for new novel biomaterial for 3-D printing, which can be crosslinked without the need of external synthetic crosslinker such as glutaraldehyde and can be dissolved with external change in stimuli was also the desired motive behind this research. Currently used biomaterials for 3D printing does not have the capability to degrade instantly when some external stimuli are applied which prohibits a lot of other applications which could have been discovered. The degradation of the 3-D printed materials under external change in stimuli opens a wide range of new application fields. One such field is patterning of microfluidic channels inside the polymer structures.

Developing the target specific drug delivery system based on biomaterial was another research motive. Oral drug delivery systems for delivering drugs and genes to the intestinal tract is a challenging area of research because of the acidic and enzymatic environment of the stomach which degrades most of the materials and gene molecules. Due to the differences in physiological

characteristics of the GI tract, a pH responsive material can be developed to deliver drugs or genes to the intestinal tract at pH 6.6 - 7.5 as compare to the acidic pH 1.0 - 3.0 of the stomach.

In this report, we have described a novel pH based sol-gel conversion mechanism of the carboxymethyl modified chitosan to present a new biomaterial which can undergo gelation in the acidic solution and can again be changed back to solution phase by adding basic solution. This property was further put into operation to engineer different materials as per end use requirements such as, Controlled drug release delivery systems, microfluidic channels and 3D printing material, which can be use for different applications like conductive 3D patterns were printed for biomaterial based sensor applications.

1.3 Objectives

The main objective of this research was to study pH responsive sol-gel conversion of the CMC hydrogel and to employ it in different applications based on the pH responsive behaviour of the hydrogel formed. These objectives can be further subdivided into the following to get an better insight into this research,

- Study the sol-gel conversion mechanism of CMC hydrogel in acid and basic solutions.
- Conducting rheological analysis to study the mechanical properties of the hydrogels.
- Preparation and analysis of peel able onion-like multimembrane hydrogel systems.
- Study the controlled release potential for drug delivery-based applications.
- Analyzing shape memory behavior based on paper origamis.
- Use chitosan as a source material for 3-D printing, which can be used to print chitosan hydrogels into different patterns.
- Fabrication and analysis of CNT-chitosan based conductive 3-D patterns for biomaterial-based sensor applications.
- Fabrication of microchannel in polymer using 3-D printed chitosan hydrogel to design patterns.

1.4 Summary of Experimental Methods and Major Findings

1.4.1 Summary of Experimental Methods

Experimental procedure of the thesis can be summarized as the preparation of CMC solution by dissolving different concentrations of CMC in water which can be phase transferred using acid solution and then using it as per end user required procedures based on the desired applications which can be described as follows,

- **Onion like hydrogel -** CMC solution and hydrochloric acid solutions were used for this procedure. Core CMC hydrogel was made by adding acid solution to the CMC solution, followed by repeated washing with D.I. water. Core hydrogel was then dipped in the CMC solution and acid solution sequentially followed by washing with water after every acid treatment. The above procedure can be repeated as per the required number of layers wanted on the hydrogel.
- **Controlled release studies –** CMC solution, hydrochloric acid solution and fluorescence were the materials used to execute the above procedure. Fluorescence was dissolved in CMC solution which was phase transferred using hydrochloric acid solution. The above prepared hydrogels entrapping fluorescence inside was then used for controlled release systems. Solutions of different pH was used for this study.
- **Shape memory behavior –** CMC, hydrochloric acid and sodium hydroxide solutions were used for developing shape memory applications of CMC solution based on the behavior of CMC in acid and basic solutions. CMC solution was applied on the one side of the paper and was phase transferred in acid solution which was then able to recover to its original shape when it was placed in the basic solution.

- **3D printing bioink –** CMC hydrogel was presented as a new bioink material which can be used for printing. The solution was printed on the substrate and was then phase transferred to a solid phase by emersion in hydrochloride acid solution.
- **E skin biosensor –** CMC and CNT solution were mixed for synthesizing biosensors which can sense body movement, breathing and speech. 3D printing was used to design the sensing material which was then used to design a wireless biosensor.
- **Microchannels –** Fabrication of microchannels was done by using 3D printing of CMC materials to design the microchannel pattern. The printed pattern was then immersed in PDMS sheet. PDMS sheet was then subjected to immersion in basic solution for dissolution of the chitosan hydrogels thereby making microchannels in the PDMS in the spaces which were earlier employed by CMC hydrogel.
- **Rheology and Dissolution tests –** Different concentrations of CMC was prepared and phase transferred with different concentrations of hydrochloric acid during these experimental procedures to determine the mechanical properties and physiochemical properties of the hydrogel systems.
- **Sol-Gel conversion test –** CMC, hydrochloric acid and sodium hydroxide solutions were used to conduct this experiment to gain an insight into the phase transition capability and conversion of the hydrogel back to the solution phase. Initially, acid solution was added to the CMC solution forming a hydrogel structure which was then dissolved back to solution by using basic solution. Acid solution was then added to the CMC solution to assess if it can again convert back to hydrogel phase or not.

1.4.2 Summary of Major Findings

One of the major findings of the project was the pH sensitive sol-gel conversion of the biomaterial, CMC. CMC solution was found to be phase transferred into hydrogel phase in the acidic pH solutions and had the conversion into solution phase in response to the basic solution. It was also observed that this property of the CMC solution was reversible and can be triggered into phase change by changing pH values of the solution. The sol-gel conversion property of the CMC solution was then exploited to make materials which can be used in wide range of applications. Synthesis of peel-able onion like hydrogel systems was one of the secondary major findings of this project which resulted in the formation of hydrogel systems with desired number of layers. These hydrogels can be used in various biomedical applications such as drug-delivery, tissue engineering, etc.

Capability of this material to be used as bioink in 3D printing is another important finding of this project. This material can be used as an alternative to currently used biomaterials, which opens some new field applications such as biosensor applications, microchannel systems, etc. Synthesis of biosensor for body movement, breathing and speech detection was one of the major applications of 3D printing capability of the CMC hydrogel.

Another secondary finding of the project was the controlled release capability of the CMC hydrogels in the basic solution which can be utilized in the intestine specific drug-delivery systems.

1.5 Thesis Layout

The current work can be categorized in the following chapters to organize the findings of the research project and to establish a link between the results and discussions of the thesis,

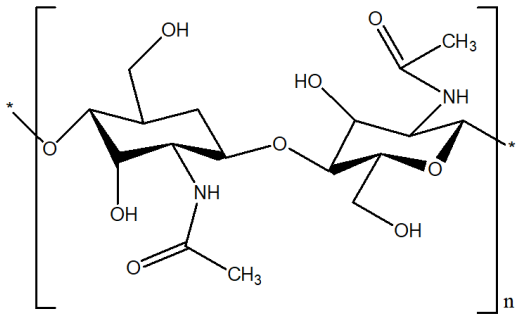
- Chapter 1 – A brief introduction of background, problem definition, objectives, methodology, and major findings.
- Chapter 2 – Presenting the general literature review planned for the research on CMC, hydrogels and stimuli responsive hydrogels, trending application fields for novel biomaterials.
- Chapter 3 – Brief exhibition of the experimental procedures and the instruments used in the research project.
- Chapter 4 – Results and discussions of the research work
- Chapter 5 – Major findings and the summary of the project.
- Chapter 6 – Propositions on the future work.

Chapter 2: Literature Review

2.1 Natural polymers

Polymers can be defined as the materials which are made of smaller monomer subunits which can be present in association with each other. Polymers can be subcategorized into two categories depending on the type of monomers, natural polymers and synthetic polymers. Natural and synthetic polymers have been widely investigated for their applications in the field of biomedical applications because of their wide range of properties which can be tunable as per end user requirements. Natural polymers are studied more significantly in terms of their biomedical applications over synthetic polymers because of their elevated biocompatibility, non-toxicity, ability to break down into components which can be utilized in the metabolism cycles by the body[41-43]. Due to the close resemblance of components in natural polymers to that of the extracellular matrix (ECM), these polymers in most cases, do not cause any immunological response in the body as compared to the synthetic polymers. Natural polymers mainly include all Polysaccharides and polypeptides which are constituted mostly of carbon derived monomers (carbohydrates). Polysaccharides mostly derived from the renewable resources like plants, animals and microorganisms are in the form of cellulose, chitosan, alginate, hyaluronic acid, etc. Starch and cellulose are the types of polysaccharides which are derived from the plants and are often regarded as plant based polymers. Cellulose is one of the most abundant natural polymer found on the earth, present in every plants and tree[44, 45]. Chitin is the main animal derived natural polymer found in the shells of most of the animals in crustacean family. Proteins are another class of natural polymers which are classified under the polypeptides[46]. Different polymers have different physicochemical properties which can be used for various interdisciplinary

applications[47]. However, natural polymers have some limitations in their properties, mainly in terms of solubility, oxidising temperature, mechanical strength and degradability[48]. These limitations must be addressed before using natural polymers for desired applications. Polymer modification is the one such way to alter the existing properties and create a new type of polymer which has the desired physicochemical properties. Depending on the type of modification, it can either be chemical modification or physical modification. Chemical modification alters the polymer structure by changing the chemical moieties present on the polymer backbone with the new chemical groups[49, 50]. Physical modifications are type of modifications in which, the surface of the polymer is altered by physical attachment[51]. Crosslinking of these polymers have also been investigated to increase the structural integrity of these polymers and in turn to improve their mechanical strength. Chitosan is one such polymer which has been derived by the chemical modification of chitin[17]. Chitosan is produced by the deacetylation of chitin, followed by addition of amine groups, which also improves the solubility of chitosan in acidic solutions as compared to the chitin which has very poor solubility[52, 53]. Furthermore, chemical modifications of chitosan leads to the introduction of novel physicochemical properties in the material[54, 55]. Depending on the substituted chemical group, chitosan with wide range of modified properties can be synthesized for different applications. Structures of cellulose, chitin and chitosan are shown in fig. 1



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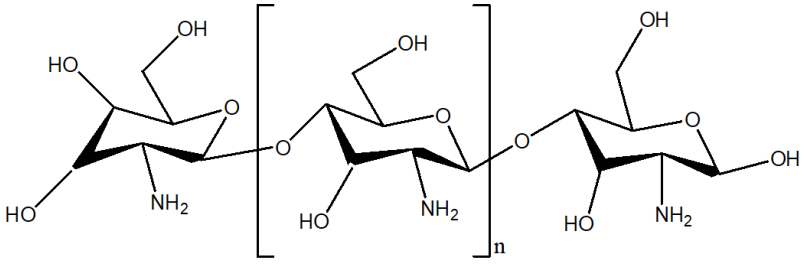

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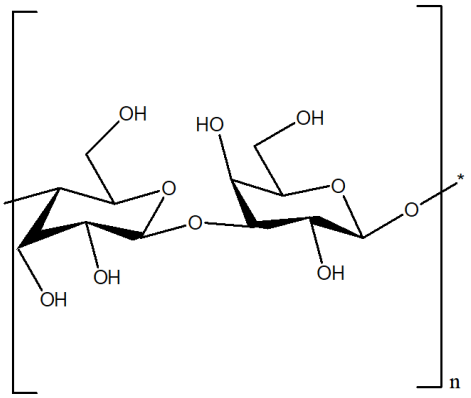


CELLULOSE

Fig. 1 Chemical structures of Chitin, Chitosan and Cellulose.

2.2 Chitosan

Chitosan is a linear polysaccharide macromolecule which contains “β 1/4-D-glucosamine and β 1/4-N-acetyl D-glucosamine units”. Chitosan can be produced by partial deacetylation of the chitin[56]. Chitin is one of the most abundantly available polymer on the planet. Chitin is mostly present in the crustacean shell, insects, certain fungi, and bacteria[22]. Chitin has the structural similarity with cellulose but differs in the chemical inertness which limits the applications of the chitin. By converting the acetamide group of chitin to an amino group, chitosan can be prepared. Chitosan is relatively reactive as compared to the chitin and can be used for different biomedical applications[57-60]. However, chitosan has a limited solubility due to it’s pKa value of 5.6 – 6, which makes it only dissolvable in the aqueous acidic solutions. This is because the amino groups of the chitosan can be protonated only in acidic pH[61]. As, chitosan can only be dissolved in acidic solutions, the neutralization step before any potential medical application may cause the change of shape, size and properties of the system and may limit their final properties, which were required in the beginning[16]. Therefore, even chitosan cannot be extensively used in biomedical applications because of its inability to get solubilized in the biological solutions.

Chemical and physical modifications of the biomaterials are the gateways through which the desired properties of materials can be achieved and altered. There are a number of chemical modifications which have been added to the chitosan backbone and have resulted in a change in many important properties and widespread the applications of chitosan which have been summarized in table 1.

Modification	Solubility	Characteristics/ Applications	Reference
Carboxymethyl chitin	Water soluble	Decreasing the adsorbing capacity of blood components, biocompatible, no antibody induction	[62-64]
O-carboxymethyl chitosan	Water soluble	Non-toxic, biodegradable, biocompatible, antimicrobial bio-activity	[65-69]
N-carboxymethyl chitosan	Water soluble	Used in medical purposes, cosmetic products and as chelating agent	[70-73]
N, O-carboxymethyl chitosan	Water soluble	Moisture retention, gel-forming, and good biocompatibility	[68, 74-77]
N-carboxybutyl chitosan	Water soluble	Good film-forming polymer, good moisture retaining agent with high bacteriostatic capability	[78-82]
N-carboxyethyl chitosan	Water soluble	Antioxidant characteristics, antimutagenic activity and better biodegradability than chitosan	[83-87]
N-succinyl chitosan	Water soluble	Very less cytotoxicity with less biodegradable inside the human body.	[88-91]
O-succinyl chitosan	Water Soluble	allows chemical functionalization to adjust water solubility and biodegradability.	[16, 92]

Table 1. Different chemical modifications of Chitosan

Carboxymethylation is one such chemical modification of the chitosan which results in the change of chemical and physical properties of the chitosan. Depending on the site of substitution in single chitosan molecule, it can either be an O-carboxymethyl chitosan with substituent positions of the D-glucosamine unit at the C-3 and C-6 or an N-carboxymethyl on the-NH 2 group. CMC changes the solubility of chitosan in biological solutions and can be dissolved in the water. The soluble nature of CMC in the biological solutions at pH value 7 or above, results in the use of chitosan in various biomedical applications such as absorbing metal ions[93], drug delivery[94], wound healing[95], antibacterial agents[96], cosmetics[97], food preservation[98], tissue engineering[99], biosensors[100].

2.3 Carboxymethyl chitosan

Chitosan is readily studied as a potential material for pharmaceutical applications but there is a difficulty in realizing it because of its poor solubility. Carboxymethylation of chitosan is done to overcome the solubility drawback and expand its solubility in a wide range of pH values[101]. Chemical structure of chitosan has different reactive groups such as amino group, primary hydroxyl and secondary hydroxyl groups which can be utilized to add different chemical modifications in the polymer to alter the physicochemical properties of the chitosan. CMC is the most widely studied water-soluble derivative of chitosan because of its ease of synthesis, ampholytic character and its wide range of potential applications[102].

Depending on the site of carboxymethylation on chitosan structure, it can be of three types: N-carboxymethyl chitosan, O-carboxymethyl chitosan, N, O-carboxymethyl chitosan[103]. There are ample procedures reported for the synthesis of CMC. The main methods found in the literature for carboxymethylation of chitosan can be summarized as follow, (i) reductive alkylation in which

-NH_2 group of chitosan reacts with the carbonyl group of aldehyde-glyoxylic acid followed by the hydrogenation with $NaBH_4$ or $NaCNBH_3$ for the synthesis of N-carboxymethyl chitosan. (ii) a direct alkylation is an approach in which monohalocarboxylic acids are used as the monochloroacetic acid for the synthesis of N-carboxyalkyl and O-carboxyalkyl chitosan derivates[104].

2.3.1 Biological properties

Cell adhesion & functioning

Through the extensive research on chitosan, it has been established that the chitosan is favourable for cell attachment by aiding in the cell morphology, proliferation and differentiation[105]. Carboxymethylation of chitosan is indicated to preserve these characteristics of chitosan[106]. Chitosan is being extensively used in tissue engineering because of its similar characteristics to extracellular matrix helping in the growth of new tissues. Controlled degradation rates are the obligatory measures for any material to be used in tissue engineering. However, slow and poorly controlled degradation rates of chitosan are the hurdles in the realization of chitosan as a material of research in regenerative medicine. Carboxymethylation of the chitosan improves the degradability of the chitosan while retaining the characteristic properties of the chitosan facilitating in the tissue engineering[106]. CMC has also been established as a non-toxic material and has been used as a blood compatibility agent on the artificial vascular grafts which hinders the platelet adhesion on the surface[107].

Antimicrobial activity

Presence of -NH_2 group in the chitosan is proposed to contribute to the antimicrobial nature of the chitosan. It is believed that due to the polycationic nature of chitosan, it interferes with the negatively charged macromolecules at the cell surface thereby interfering with the cell permeability properties[108]. CMC possesses the similar antimicrobial activity inhibiting the growth of wide variety of fungi and bacteria. O-CMC was found to have the higher antimicrobial action because of the higher number of -NH_3^+ groups in OCMC as compare to the chitosan responsible for the antimicrobial property. Increase in the M_w of the polymer also increase the number of amine groups in chitosan and CMC[109]. However, increase in the intrinsic flexibility of chains because of the higher M_w decreases the number of amine groups available for the antimicrobial action. Therefore, a balance between both these factors are required for the antimicrobial activity[110].

Antioxidant activity

Active hydroxyl and amino groups in the chitosan, which takes part in the free radical scavenging, are being investigated for the antioxidant activity of the chitosan[84]. It has been discovered that amount of active hydroxyl, amino and amide groups in chitosan along with the molecular weight of the polymer are the parameters responsible for the antioxidant characteristics of the chitosan and its derivates[111]. According to a recent study, decreasing the molecular weight of chitosan and its derivatives increases the antioxidant activity because of the partial destruction of intramolecular and intramolecular hydrogen bonds[112].

Apoptosis inhibitory activity

Chitosan and chitosan derivates have also been studied for their potential apoptosis inhibitory activity. Chitosan has been reported for its applications in cancer treatment through their growth inhibitory effect on bladder tumouric cells[113]. It has also been indicated that IL-1β-induced chondrocyte apoptosis can be inhibited by changing the amount or dose of the CMC, which can be attributed to the protected mitochondria function, decrease in the levels of nitric oxide and reactive oxygen species[114]. It has also been demonstrated that CMC reduces the postoperative adhesion formation in an animal model through a cardiac injury and an abdominal surgery study[115].

2.3.2 Physicochemical properties

Water Solubility

Addition of carboxymethyl groups to chitosan is performed to enhance the water-soluble ability of chitosan in water at neutral pH values meanwhile imparting novel functionalities to the chitosan. Moisture absorption and retention by CMC has been experimentally shown in relation to the amount of deacetylation and degree of substitution[113]. It was stated that, the hydrogen bonds between water and polymeric chains and the presence of COO^- group for solubility of CMC in water. However, the intermolecular H-bonding of CMC and the electrostatic repulsive forces between the polymer chains are also known to be responsible for the aggregation of CMC in water[116]. In another study, it was observed that the rheological properties of the CMC were affected by the modification of hydrophobic as well as hydrophilic groups[117]. It was also observed that, the ionic strength did not have any significant affect on the CMC in terms of self-

aggregation. Carboxymethyl derivation of chitosan has been found to produce more favourable polymer materials with moisture retention capabilities.

Chelating and adsorption properties

Chitosan has been found to have excellent chelating and sorption properties. Due to the higher amount of hydroxyl groups and amino groups in the chitosan, it helps it to adapt to a different configuration[118]. The flexibility of chitosan structure is responsible for the formation of complexes with metal ions, making them an excellent chelating material. In one report, CMC and crosslinked CMC polymer with Cu (II) was used as a template for the selective adsorption of Cu (II) from the solution consisting of three different types of metallic ions[119]. Similarly, in another report adsorption of Fe (III) ions on the CMC hydrogel has been documented in which the adsorption time was found to be quick, just below 20 minutes. It was also demonstrated that chelating of Fe (III) ions took place with amino group, hydroxy group and carboxyl group of the CMC[120].

2.4 Hydrogels

In the last decade, hydrogels have been the prime research interest of the material scientists all over the globe which have led to the significant progress in designing, synthesizing and their applications in many biological and biomedical fields. Hydrogels can be defined as the chains of hydrophilic polymer network crosslinked together which tends to absorb and hold water up to thousand times of their own original weight. The absorbed water is stored in the spaces between the polymer chains. Presence of crosslinkers between the polymer chains prevents chains from dissolution upon contact with water and thus allows water to penetrate the spaces between the polymer network. This property also contributes towards to the biocompatibility of the hydrogels,

as most of the tissues have ~90% water content and therefore, these gels have similar resemblance to the natural human tissues[121]. Biocompatibility and degradation into minimal toxic materials are the fundamentals of biomedical research. Therefore, degradation of the hydrogels must lead to the non-toxic products which can be excreted or utilized by the human body. The excellent water absorbing capacity and tunable degradation mechanisms of the hydrogels have expanded their applications in various medicinal areas of applications like drug and protein delivery systems, materials for 3D printing, tissue engineering and regenerative medicine, and bio-nanotechnology[122-125].

Hydrogels can be subclassified into various categories depending on different basis. Depending on the materials used for these polymer chains, the hydrogel can be classified into two categories, synthetic hydrogel systems and natural hydrogel systems. Synthetic hydrogels are made from synthetic polymers like poly-lactic acid (PLA), poly-acrylic acid (PAA), poly-ethylene oxide (PEO), etc. On the other hand, if the polymer used is a natural one like alginate, chitosan, cellulose, etc., the hydrogels obtained can be classified as natural hydrogels[126]. Vital property of natural hydrogel is there significantly higher biocompatibility than the synthetic hydrogels. Synthetic hydrogels have been sometimes reported to have significant reactions with the tissues within the human body. Synthetic hydrogels, despite being less biocompatible, are widely used in biomedical applications due to their high strength and control over degradation rate[127]. Polymeric composition used for synthesis of hydrogel defines the next class of classification for hydrogels, homopolymeric, copolymeric and multipolymer interpenetrating hydrogels. As the name suggests, homopolymeric hydrogels are made of single type of monomer as a basic structural component of the hydrogel system. If the hydrogel contains two types of polymers, it is classified as copolymeric hydrogel with one of the polymeric chains being a hydrophilic polymer. Polymer chains in the

hydrogels can either be arranged randomly or in any specific pattern. Interpenetrating polymer hydrogel which contains more than one polymer is an imperative kind of hydrogel where two independent crosslinked polymer chains are present in the same hydrogel. The polymeric chains in interpenetrating polymeric hydrogel can either be crosslinked with each other or can be present independently in the hydrogel system. Crosslinking is an integral part of hydrogel as it prevents the hydrophilic polymeric chains from dissolution in the aqueous solutions, thus providing the structural integrity to the hydrogel[128]. Hydrogels can further be classified into two categories based on the nature of the crosslinking network, physical or chemical crosslinks. Chemical crosslinked networks are the ones which are crosslinked permanently, while the physical crosslinks have transient junctions which can either result from chain entanglements or physical interactions such as ionic interactions, hydrogen bonds, etc.[127] Finally, hydrogels can also be classified based on the physical structure of the networks such as amorphous, semicrystalline, hydrogen bond networks, supramolecular structures and hydrocolloidal aggregates[129].

2.5 Stimuli responsive hydrogels

Stimuli responsive hydrogels or 'smart hydrogels', are defined as the type of hydrogels which possess capability to respond to the external stimuli. The response can be in the form of undergoing certain changes in their swelling- deswelling properties, interconnected networks, tensile strength and in their permeability[130]. These stimuli can be subdivided into two main categories, physical and chemical stimuli. Physical stimuli can be described as any source of environmental energy such as pressure, light, temperature, electrical changes, mechanical force, etc., which can alter the inter and intra molecular interactions between the hydrogel network. On the other hand, chemical stimuli can be a change in pH value, ionic species and chemical compounds which have tendency to alter interactions inside the hydrogel at the molecular level, which might include interaction

between polymer chains and solvent and within different polymer chains inside the hydrogel. There is another class of smart hydrogels which is called dual stimuli responsive hydrogels. These hydrogels have tendency to respond to two different stimuli in one hydrogel systems[131]. Stimuli responsive hydrogels have been one of the attractive biomaterials for applications in the field of health sciences[132-135].

pH responsive hydrogels

When a polymeric hydrogel has potential to accept or donate protons as a response to the external changes in pH, then the polymeric hydrogel can be classified as pH responsive hydrogel[136]. The degree of ionization, pK_a or pK_b are one of the few properteis known to be drastically changed at a specific pH in a pH responsive hydrogel[137]. This sudden change in ionization of the polymer groups leads to a volume change as a responsive action due to the initiation of electrostatic repulsions between the ionized polymer groups. This leads to an increase in the swelling force because of the osmosis effect. Based on the different pendant groups present in the hydrogels, pH responsive hydrogels can be divided into two sub categories, anionic and cationic hydrogels. Anionic hydrogels contain polymeric groups like carboxylic and sulfonic acid groups. In anionic hydrogels, the deprotonation occurs when pH is higher than the pK_a value, resulting in the ionization of the polymer and in turn causing an increase in the swelling of the hydrogels[138, 139]. Whereas, in case of cationic hydrogels, the presence of amine groups causes ionization, when the pH of the external solution is below pK_b, which in turn causes an increase in swelling because of the increase in electrostatic repulsive forces[140, 141]. Because of their tendency to go through drastic changes affecting their volume, elasticity and mass in response to the changes in pH value, this class of hydrogels has found applications in wide range of biomedical sciences ranging from drug delivery systems to sensors and actuators[142].

It was reported in a study, that alginate-N, O-carboxymethyl chitosan (NOCC) hydrogel beads with coating of chitosan can be used for colon targeted drug delivery systems[140]. It was observed that the hydrogels swelled higher at pH 7.4 as compared to pH 1.2, concluding the pH sensitive nature of the hydrogels. In another study, they reported a superabsorbent pH sensitive starch poly (sodium acrylate-*co*-acrylamide) based hydrogels which were studied for the release rates of drug in imitated stomach and intestine pH values[143]. It was also observed that hydrogels were able to release higher dosage of the drug at ph7.4 as compared to the pH 1.2.

In a study, they [144] reported the fabrication of pH responsive hydrogels using gold nanoparticles, which were used for developing a pH sensor with high sensitivity and very quick response time. It was demonstrated that a plasmon resonance shift of 50 nm was observed by decreasing pH from 5 to 2, which caused 70 % increase in the film thickness. In another study[145], they reported an actuator which was made by photo patterning and was developed by using N-isopropyl acrylamide, acrylic acid, and polyethylene oxide diacrylate films. Swelling behaviour of individually patterned hydrogel was found to be responsible for the fabrication of actuators. Change in pH of the system and ionic factors, consequently caused the folding and unfolding of the bilayer structures

Temperature responsive hydrogels

When the swelling and deswelling behaviour is observed in the hydrogels as a response to change in the temperature of the external environment, such hydrogels are defined as temperature sensitive hydrogels. Further, temperature sensitive hydrogels can be categorized as positive and negative temperature sensitive hydrogels[137, 146]. There is another type of temperature responsive hydrogels, which is same as the other two types but the difference being that these hydrogels undergo a sol-gel phase conversion as a response to changes in temperature.

Positive temperature responsive hydrogels are the type of temperature responsive hydrogels which are based on the upper critical solution temperature (UCST)[147]. These hydrogels were reported to contract (deswelling) when the temperature is below UCST and tend to swell when the temperature is raised higher than UCST. This retrogressive behaviour of these hydrogels at low temperature can be attributed to the complex structural formation caused by the hydrogen bonds at low temperature and dissociation of the structure at high temperature thereby leading to swelling. The most widely studied positive temperature sensitive hydrogels are the ones that are formed by IPNs. A study reported the positive temperature responsive hydrogels based on polyacrylic acid and polyacrylamide or P (AAm-co-BMA)[148]. Increase in the content of BMA was shown to change the transition temperature from lower to higher value.

Negative temperature responsive hydrogels have their responsive behaviour based on low critical solution temperature (LCST). These hydrogels demonstrate shrinking behaviour when the temperature rises above LCST and on the other hand tends to swell with the decrease in temperature than LCST. The LCST temperature can further be shifted by addition of ionic copolymer or by altering the solvent composition[149]. The interaction of fluid or water with hydrophilic part of the polymer at lower temperatures leads to increase in swelling by the formation of hydrogen bonds between water and polymer molecules[150]. On the other hand, when the temperature is higher than the LCST, the interaction between hydrophobic parts of the polymer are stronger thus leading to the shrinkage of the hydrogel. It was reported by a study[151], that in case of PVP/PNIPAAM copolymeric hydrogels, the release profile of drug was slower when the temperature was above the LCST. This negative temperature responsive hydrogel was based on the presence of hydrophobic and hydrophilic components of the polymer.

Thermo responsive hydrogels are very similar to positive and negative temperature hydrogels. The temperature sensitivity in the other types of hydrogels is observed due to the hydrogen bonds, whereas in case of thermo responsive hydrogels, which are not covalently crosslinked, they undergo sol-gel phase transition contrary to the swelling and deswelling behaviour. The most widely commercially available thermo reversible hydrogels are commonly known as Pluronics and Tetronics[152] which are being used in various food additives or preservative, medical ingredients and agricultural products approved by FDA and EPA.

Light responsive hydrogels

Light responsive hydrogels are the type of hydrogels in which the stimuli governing the changes in the hydrogel is light. Light has been known to stimulate various responses in the hydrogels, such as crosslinking cleavages, diffraction shifts, molecular (nano and macro) uptake and release, and detection of ions[153-156]. Light is a most widely studied type of stimuli for stimuli responsive hydrogels as different parameters of light like wavelength, spatial and intensity can be changed with ease which makes it most controllable stimuli[157].

The same principle has been deployed for the synthesis of hydrogel and also for the degradation of the hydrogel upon the projection of light. In these hydrogels, crosslinks used for the synthesis of the hydrogels are based on the photoresponsive crosslinks and these photo degradable crosslinks are very critical for developing photoresponsive hydrogels[153]. It was reported that, by using the acrylates connected with a nitrobenzyl ether photo degradable moiety to the PEG groups, they were able to add the photodegradable response to the PEG based hydrogels.

One of the other methods used to introduce the photoresponsive behaviour into the hydrogels is the cis-trans isomerization of azobenzene[158]. In one study[159], it was reported that cis

conformation of the hydrogels containing azobenzene allowed the widening of the pores and in turn haid in the water to be released from the hydrogel. In another study[160], they reported the hydrogel made of NIPAM and 11-(acryloyloxyundecyl) trimethylammonium bromide which was made by using gamma radiations. It was observed that when the temperature was lower than the LCST, the azo group was able to be isomerized as a response to the light stimuli, however the azo group was not able to convert back among the isomers because of the steric hindrance caused by the shrinking of the hydrogel. This technique of using temperature to control the gel properties can be potentially used to build smart hydrogel systems.

Glucose responsive hydrogels

Hydrogels which exhibits various responses to the presence of glucose in the surroundings are classified as glucose responsive hydrogels. The main working principle of glucose responsive hydrogels is based on the enzyme substrate reaction, where a change in pH value is observed and thus a pH sensitive hydrogel can be used which can respond to these pH changes[161]. These hydrogels have been reported for their use in insulin delivery in the diabetic treatments by mimicking the natural insulin release based on the glucose level in the external environment. The glucose responsive hydrogels can be subdivided into two categories based on their sensing mechanisms, glucose oxidase based hydrogels and concanavalin A based hydrogels.

The glucose oxidase based hydrogels works by a reaction of glucose with glucose oxidase (GOD), which forms a gluconic acid (GlucA), which causes a decrease in the pH value of the system[162]. This decrease in pH further acts as a stimulus for the hydrogels to swell or deswell depending on different polymers. In recent studies[163, 164], they reported the glucose responsive hydrogels based on the glucose oxidase enzyme consisting of polymers, Hydroxyethyl methacrylate (HEMA)

and Polymethyl acrylate (PMA). It was observed that local pH of the system was decreased by the conversion of glucose in presence of oxygen by the glucose oxidase to gluconic acid, resulting in increase in the swelling of these cation based hydrogels thereby triggering the release of the insulin.

The Con A based glucose responsive hydrogels are based on the competition of free glucose molecules and glucose insulin conjugates bounded with Con A. Con A is a type of protein which has the ability to bind to the glucose[152]. When free glucose diffuses into the hydrogels, the surrounding tissues are deluged with glucose-insulin conjugates, where they are able to display their bioactivity based on the levels of glucose in the region[130].

Antigen responsive hydrogels

Hydrogels based on the antigen-antibody interactions are classified as antigen responsive hydrogels. These hydrogels are developed by adding antigens to the hydrophilic polymers, which can transport molecules to the target site. The working principle behind these gels is that when the free antigens are not present, the intrachain antigen-antibody conjugation in the polymeric network is enhanced which results in the shrinkage in the structure of these hydrogels[165]. These antigen sensing hydrogels have been used in various biomedical applications such as biomolecules, drug and protein delivery systems and biosensors[166]. An antigen responsive hydrogel was reported in which the antigen-antibody interaction was used in crosslinking the polymer network[167]. It was demonstrated that the free antigens triggered a change in the volume of the hydrogel because of the breaking of the non-covalent crosslinks which can be used for delivery applications.

Humidity responsive hydrogels

Some hydrogels have been reported for high sensitivity towards changes in the humidity in external surroundings. In a recent study[168], they reported a humidity based sensor hydrogel, in which dapoxyl sulfonic acid was used as a fluorophore. It was observed that a shift in DSA's fluorescence was caused by the absorbed water in the hydrogel which leads to change the polarity. The detection of this shift was the working mechanism behind this humidity sensors. It was also reported that, the ground and excited state of the fluorophore can be stabilized to different degrees by fluorophore and solvent molecules interactions. This process is also known as Solvatochorisim. In another study[169], a hydrogel based on PNIPAM and titanium(IV) isopropoxide was reported, which demonstrated humidity sensitivity based on the changes in refractive indexes.

2.6 Hydrogel applications

Hydrogels are one of the recent marvels of the science, with significant properties which make them a material of interest in engineering, biology and pharmaceutical sciences. Stimuli responsive hydrogels can display significant changes as a response to even slightest of change in the environment and have been used for their wide range of applications in drug delivery, agriculture, medicine, pharmaceutical and biomedical sciences[142].

Biomedical applications

Stimuli responsive hydrogels have been widely used in medical implants, prosthetic organs, biorobots, artificial muscles, diagnostic devices, etc.[170-172]. Applications of hydrogels in artificial muscles were reported[172], in which they demonstrated the ability of hydrogels to convert electrotechnical stimuli into mechanical responses. The hydrogels were able to contract

and relax as a response to external physicochemical stimuli. In another study[173], they reported a hydrogel based on PEG-PLGA graft copolymer which was able to diagnose and treat cancer cells based on the molecular recognition and stimuli responsive properties. They purposed that these hydrogels can be used as a drug delivery system as they could cross the cell membranes targeting specific tissues. These hydrogels were reported as a smart hydrogel with potential applications in a variety of research areas. Synthesis of clear PPO-PEO hydrogels at room temperature was also reported with potential applications in wound healing[174]. It was demonstrated that these hydrogels were able to cover the spaces in the wound and were able to protect the wound area from microbial infection. These hydrogels were also reported to be flexible, durable and are permeable to moisture and other molecules.

Hydrogels have been widely studied for their application in tissue engineering as a scaffold material for 3-D cell culture. The ability of hydrogels to hold high water content and their mechanical properties similar to the tissues are the driving force behind their use in tissue engineering. In one study[175], they reported a hydrogel for tissue engineering applications. They constructed a self healing hydrogel composed of cellulose scaffold with multi stimuli responses and better mechanical properties, which were used for 3D cell encapsulation and cell culture of L929 cells. Also in another study[176], they synthesized a hydrogel by oxidation of CS, GO, and DA mixture. It was found to increase the beating rates of the tissues to double than that of non - treated tissue culture polystyrene. Therefore, it can be potentially used in electroconductive tissues such as cardiac and neural tissues.

Pharmaceutical applications

Controlled drug delivery is the most important pharmaceutical application of hydrogels, widely ranging from liver, kidney, stomach, intestinal tract, brain, nervous system and tumor specified delivery systems[177]. The porous structure of hydrogels plays a very important role in loading and protecting the drug molecules from external environments and can deliver at the targeted site. Ability to tune release rates and biodegradability along with biocompatibility are among the significant properties which have contributed to the hydrogels for their use in drug delivery applications[178].

In a study[179], they reported an injectable hydrogel made of Polyethylene oxide -*b*- poly propylene oxide -*b*- polyethylene oxide triblock copolymers (PEO–PPO–PEO) (Poloxamer) for target specified control delivery of anesthetic agent lidocaine. This copolymer was also found to be suitable for commercially hospitalization based applications of the Poloxamer. Other research studies[180-182] about improving the Poloxamer based delivery systems introduced the covalent crosslinking with another chemcial group, like ethoxysilane, amine, or carbohydrates to increase the stability of polymer in the aqueous solutions thereby increasing the diffusion rates and duration of drug release. In another study[183], it was reported that the synthesis of a temperature responsive hydrogel of PNIPAM-PEGDA can be used for its applications in pharmaceutical sciences. It was observed that the hydrogels displayed excellent loading efficiency with temperature sensitivity for site specific delivery. In another study[184], they reported an insulin release system based on poly (2-hydroxyethyl methacrylate-co-N, N-dimethyl amino ethyl methacrylate), also known as poly(HEMA-co-DMAEMA), with encapsulated catalase, glucose oxidase and insulin. It was demonstrated that when the hydrogel was brought in contact with physiological fluids, diffusion of glucose to the hydrogel network lead to the formation of gluconic

acid by glucose oxidase, which started the swelling of the pH rsponsive hydrogel and lead to the insulin release into the physiological environment.

Sensing applications

Stimuli-sensitivity of the hydrogels can be used for the development of sensors based on electroconductive hydrogels for various sensing applications. The electroconductive polymers are both electrically and ionically conductive polymers which tends to provide a non-toxic interface between the device and the living tissues for biosensing applications[185]. In a recent study, a turn on protease biosensor based on the auto inhibited coiled coil switch was developed, which was used in protein detection and could be utilized for specific labelling of proteins in the cells[186]. In another study[187], an electroconductive hydrogel based on poly (HEMA) and poly(aniline) were reported for biosensing applications by the adding recombinant cytochrome P450-2D6. It was observed that these hydrogels displayed faster switching than their pure electroconductive polymer counterpart.

Agricultural applications

Recently, hydrogels have been investigated for controlled release of nutrients and fertilizers to the plants[188]. A significant quantity of fertilizers which are provided to the soil gets washed away or leached away due to the highly porous nature of soil beds. Therefore, several different hydrogel systems based on chitosan, pectin and carboxymethylcellulose have been investigated for preparing a controlled fertilizer release into the soil[189]. Another study, indicated that hydrogels are essential for plantation forest establishment by helping in the moisture retention in a specific soil[190]. It was observed that the moisture retention increases the soil attributes like aeration, temp. control, transporting nutrients, water intake and transforming soil which all affects the crop

productivity. In another study[191], they reported a cellulose dervied hydrogel for controlled releasing of water and nutrients into the soil in dry soils. It was also demonstrated that the hydrogels could release the water in relation to the soil dryness, thereby keeping the soil humid for a longer time.

Applications in food packaging industry

Biopolymers are now one of the most developing packaging materials because of their eco-friendly nature. Many researchers and industrial companies have exploited the ecological advantages of these materials to develop food packaging materials. In general, different types of biopolymers are mixed together and are used to design a food packaging material or are sometime used as a filler material in the polymer matrix[192]. Every specific biopolymer has distinguished properties which can critically influence the properties of the complex attainable from these mixtures. A food packaging material based on a composite of starch and cellulose in the form of films was reported with high tensile strength[193]. In another study, an antimicrobial packaging material was demonstrated based on hydrogels made of chitosan and gelatin made by solvent-casting method[194]. Edible packaging material based on gelatin and alginate was also synthesized by extrusion method with excellent oxygen barrier properties[195].

Applications in cosmetic industry

A large amount of available of cosmetics products in the cosmetic industry, contains hydrogel systems which can be described as the new generation of cosmetic products. Chitosonic® Acid and carboxymethyl hexanoyl chitosan are some of the important biomaterials that are being used in cosmetic industry[196, 197]. These materials are acceptable for there use in cosmetic products by the Personal Care Products Council with the INCI (International Nomenclature of Cosmetic

Ingredients). The Chitosonic® Acid is a novel water-soluble chitosan based material with good hydrophilic-lipophilic ratio. It has also been reported to have significant antimicrobial properties against different microbial infections. Also, Chitosonic® Acid have good water retaining properties thereby helping in hydration. Therefore, Chitosonic® Acid is being widely used in cosmetic industry as it has good compatibility with other ingredients too.

Applications in separation technology

Hydrogels are used as separation materials for purification of water pollution. Adsorption is used as an effective method to remove harmful dyes from the water bodies and have the significannt advantage of being reusable and biocompatible. Different studies have prepared membranes with increased adsorption and desorption capability, higher separating efficacy and enhanced adsorption quantities[198]. Hydrogels have also been studied for the removal of heavy metal ions, which are highly toxic and are non-biodegradable[199].

2.7 Sol-gel phase transition

Hydrogels are generally developed by physical or chemical crosslinking of a distinct type of polymers which have a tendency to uphold significant amount of water between them while keeping their mechanical and physical characteristics intact. One recently developed method for the hydrogel formation is an in-situ hydrogel synthesis pathway in which hydrogel is formed by either photopolymerization[200] or by the phase transition[201]. This technique of hydrogel synthesis increases the range of applications of these hydrogels in biomedical sciences[202]. These in situ forming hydrogels undergo changes from being a clear polymeric solution to viscoelastic gel because of external stimuli such as pH and temperature[203, 204].

Phase transition can be categorized as the most interesting and important kind of hydrogel formation methods owing to their change in phase from solution to gel phase without the need of any external chemical reagent or reaction. The elimination of the need for chemical crosslinker is highly advantageous for the hydrogels in terms of biocompatibility and for their use in pharmaceutical and biomedical sciences[205].

Sol to gel and gel to sol phase conversion properties of some polymers are usually exhibited in response to an external change in temperature or pH[206]. Block copolymers of polyethylene oxide-block-propylene oxide-block-ethylene oxide (PEO-PPO-PEO) are one of the most commonly commercially available thermal responsive polymer which undergo a phase transition as a response to the thermal changes[207]. These polymers change from gel to sol phase and sol to gel phase when the critical polymer concentration is met. More recently, other copolymers of polyethylene glycol and polylactic/polyglycolic acid have also developed with sol-gel phase transition mechanisms[208-210]. The solutions of these thermal sensitive polymers can be converted to gel phase at body temperature (37°C) from free-flowing liquid phase at room temperature. Another method of in situ hydrogel formation is phase separation, which is also based on the sol-gel conversion. In this type of methodology, there is a change in the solubility of the polymer solution which can be induced by a change in the temperature or pH. It is a type of hydrogel synthesis procedure, in which secondary bonds are used like hydrogen bonds, electrostatic charge distributions or hydrophobicity based associations[207].

The phase transition can also be achieved as a responsive mechanism to the changes in pH values. pH induced phase transition is usually achieved by modifying the polymers with pH sensitive crosslinkable chemical group on these polymers[205]. This kind of phase transition is observed in the polymers which have a tendency to either acquire or give away protons as a consequence of

alteration in pH of the surrounding system. Protonation and deprotonation of these functional groups by acidic or basic solutions induces the sol to gel and gel to sol phase transitions in these polymeric solutions. Chitosan derivates are the examples of such polymers which can undergo sol to gel phase transitions based on pH changes because of the presence of amine groups and carboxylic groups on the polymer chains. Chitosan is a cationic polymer which exhibits a sol to gel phase transition when pH changes from acidic to neutral[211]. Deionization of chitosan is caused by the increasing pH, which helps in the formation of physical junctions of hydrogen bonds in the polymer solution[212, 213]. In a recent study, a sol-gel phase transition hydrogel was formed based on chitosan and sodium bicarbonate ($NaHCO_3$)[213]. These hydrogels were observed to undergo a phase transition in response to increase in pH by reaction of $NaHCO_3$ which is a weak base thereby emitting CO_2. These hydrogels were foreseen to have a great potential in biomedical applications as there was no external crosslinking agent associated with hydrogel formation.

2.8 3D Biomaterial printing

3D biomaterial printing has been established as the emerging research technology with the potential to transform medical science. There has been a recent boom in applications of this technology in healthcare sector because of the new potential biomaterials which have been discovered and with a decrease in the associated cost of the fabrication process[214]. There are several different types of 3D printing technologies available, which can be utilized for biomaterial based 3D printing such as inkjet deposited printing, photo cured stereolithography (SLA)[215], selective laser sintering (SLS)[216], fused deposition modeling (FDM)[217], direct extrusion of gels, solutions, colloidal and non-colloidal suspension[218-220]. However, there are limitations to SLA, SLS and FDM based 3D printing techniques for their applications in printing biomaterials because of the extreme conditions required for printing such as high temperature, intense optical

energies and strong solvent bath. This leaves inkjet and extrusion based printing technologies as the most suitable candidates for printing biomaterials.

However, there are certain drawbacks associated with inkjet based 3D printing technology such as they are not able to print suspensions or solutions with higher viscosity which restrict their use for printing bioinks, viscous polymers or cell based components. On the other hand, extrusion based 3D printing can extrude even high viscosity bioinks and has been successfully used in printing hydrogels, polymeric and ceramic solutions for all types of tissues and ECM components[121]. This methodology provides affirmative benefits over other printing techniques in terms of wide range of application, easily usable, precise printing of complex geometries and multiple solidification methods[221].

2.8.1 Extrusion based 3D biomaterial printing

This type of 3D printers basically operates by using an air pressure based actuators or syringe type device which feeds material through a cartridge into a nozzle or needle, through which is deposited on the substrate. The extrusion method is commonly compatible with wide range of materials followed by the curing step at the end of the printing procedure. The Most common examples of curing strategies currently being used are photo-crosslinking using photo initiators and UV light[222], temperature changes, varying the pH or ion concentration[123, 223]. In recent years, there have been some modifications to the basic single nozzle extrusion based printers in terms of multi headed 3D printers which have got the ability to print more than one material at the same time on the same substrate. Printing complex geometries is the main advantage of using multi head printers which commonly requires a sacrificial layer of the polymer to support the previous layers as each layer can be made on top of the previous layer.

Like every other 3D printing technique, extrusion based 3D printing of hydrogels comes with some advantages as well as some disadvantages as compared to the other printing techniques. The major advantage of extrusion based printing is their ability to print high fraction polymers and hydrogels containing high cell densities[224]. However, shear stress is the major concern during the extrusion based fabrication process as this can cause cell death during the printing of cell laden hydrogels[225]. In recent studies, the issue of cell death has been countered to an extent by the development of new shear thinning bioinks which have the ability to increase cell life by reducing the shear stress which is applied to the cells during extrusion process[226].

2.8.2 Applications of 3D biomaterial printing

Soft tissue mimetics are used to provide better modelling conditions for the tissue engineering methods which can be used for the assessment of toxicity of several agents in liver tissue model. It was reported in a study in which they demonstrated the ability to fabricate liver tissue with the help of 3D printing, which provided a foundation for fabricating larger tissues which were made of three different cell types[227]. These 3D printed tissues were further utilized for assessment of potential liver damage caused by the drugs. These soft tissue models have a potential role in the designing and developing the in-vitro disease models and drugs testing models.

Multi material printing has gained an influential research interest in 3D printing, which utilizes the synthesis of scaffolds for tissue engineering based applications by deposition of different layers with the material being deposited on one layer after the another. Recently, a research group demonstrated a new technique to develop a scaffold based on more than one material[228]. It was reported that the structure was made by depositing each layer with certain overlapping to maintain the structural properties of the scaffold. In another study, they reported a different route for the

development of more complicated scaffold structures with different interior geometry and composition based on the rapid deposition of multiple materials[229]. It was illustrated that these complex scaffolds printing technique could fabricate scaffold using up to seven different materials simultaneously with spatial deposition of these hydrogels.

Another application of 3D biomaterial printing is the development of bone substitute scaffolds for hard tissue engineering applications. In a recent study, a chitosan-HA hydrogel for bone tissue regeneration applications was 3D printed[230]. These scaffolds had higher elastic modulus and had higher bone filling material as compared to conventional printed materials.

2.9 Intestine specific drug delivery

Oral administration of the drugs, genes and proteins is the predominant and most convenient route for drug delivery in the patients as it doesn't require any trained personnel as compared to other delivery forms. However, several drugs, especially the protein based drugs like insulin would get degraded because of the harsh acidic and enzymatic conditions of the stomach[231]. The Gastro-intestine tract (GI tract) mainly constitutes of organs such as the esophagus, stomach, smaller intestine and larger intestine. The targeted release of drugs to point of interest parts of the Gastro intestinal tract is based on the physiological difference between the different sections of the GI tract and on the time, it takes to transit from one section to another.

The pH of Gastro intestinal tract varies with every organ, the stomach being highly acidic (pH 1-3) and this acidity decreases as we reach intestine (6.6 – 7.5)[232]. This change in pH is caused by the short chain fatty acids in the intestines arising from bacterial fermentation of polysaccharides. The variation in pH has been exploited by the researchers to deliver drugs/genes to the intestine by using pH sensitive drug delivery systems. Li et. al reported a chitosan derived intestine specific

drug-delivery system which was based on the pH triggering release mechanisms[233]. The acid labile insulin was delivered in the intestine by using L-valine modified chitosan-based multifunctional nanocarriers. In another similar study, Hou et. al reported a hydrogel system based on graphene oxide and polyvinyl alcohol with pH sensitive release of drugs in the intestine[234].

Chitosan based intestine specific drug delivery systems have been studied widely due to their favourable biological properties like nontoxicity, biodegradability and abundant availability in nature[52]. Chitosan is soluble in acidic conditions, however, can get precipitated at higher pH range. Therefore, to use chitosan in intestine targeted drug delivery systems, an external layer of polymer or chemical modification is required[235]. In these cases, the external polymer layer is degraded in basic conditions of the intestine and thereby releasing chitosan-drug solution in the intestine, where chitosan is degraded, and the drug is released.

Gene therapy is a biomedical technique used for reversing the course of many hereditary diseases and diseases caused by the presence of viral genes. Gene therapy can also be used for the treatment of diseases which are caused by genetic mutations or genetic disorders[236, 237]. Gene delivery is carried out mainly by viral vectors because of their higher efficiency and a wider range of cells which can be targeted by the viral vectors[238]. However, because of the immune responses caused by the viral vectors, a non-viral gene delivery system are being used for the gene therapy based treatments[239].

Chitosan based materials can be used as non-viral gene delivery systems because of the catatonically charged groups of chitosan, which forms polyelectrolytic complexes with the negative charge carrying plasmid DNA. It was demonstrated in a study that, plasmid DNA can be delivered to intestinal tract by using chitosan and depolymerized chitosan oligomers[240]. In

another study[60], a plasmid DNA carrying a dominant gene for peanut allergy reaction was orally delivered by complex made of chitosan and DNA nanoparticles. Reduction in allergen induced anaphylaxis was observed in the mice as compared to the no effect in the mice treated with naked DNA as a control.

Hence, based on the physiological environment of the GI tract, we can design a delivery system based on pH sensitivity. However, the main obstacle that needs to be addressed is the delivery system should be able to maintain their structural integrity in acidic and enzymatic environment of the stomach and should be degraded or dissolved by the basic environment of the intestinal tract.

Chapter 3: Experimental Procedures

3.1 Introduction

This chapter describes the experimental procedures followed in the current research project. It contains the materials used in the project, experimentational method followed for the synthesis of the material and for the characterization tests.

3.2 Materials

Carboxymethyl chitosan used in the project for preparation of CMC solution was purchased from Xi'an Lyphar Biotech co. ltd., China (batch no. Lyph20160505) with 82% degree of substitution and 96.1% DAC degree. Hydrochloric acid (HCl) was purchased from Anachemia (assay- 36.5 - 38.0%), Sodium hydroxide (NaOH) was purchased from Fischer scientific, Glutaraldehyde (25% aq. Soln.) and Iron (III) chloride (anhydrous) ($FeCl_3$) were purchased from Alfa Aesar, Whatman filter paper 4 used for shape memory origamis was purchased from VWR, Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) and Ammonium Hydroxide (NH_4OH) which were used for the synthesis of magnetic nanoparticles was purchased from Sigma-Aldrich and BDH, VWR respectively. Polydimethylsiloxane (PDMS) was purchased from Tianying (Dongguang, China). PDMS sheets were prepared by following the curing protocols stated by the supplier. Double deionized water was directly taken from Direct-Q® 3. All chemicals were used as bought without further purification. Miscellaneous stationary items (water paint, tapes) used in the project were bought from the University book store. Other miscellaneous lab equipment (gloves, pipette tips, etc.) were purchased from VWR.

3.2 Preparation of Chitosan solution & Hydrogel

For all the experimental protocols, a stock solution of CMC was prepared and used as per requirements. Briefly, 30 mg/ml solution was prepared by dissolving CMC in D.I. water under continuous stirring for about half an hour at room temperature. This solution was then used for the following experimental procedures. For hydrogel formation, the above prepared CMC solution was first frozen at -20 C for 1 hour. The solution was frozen prior to gelation because of the rapid gelation which inhibits the synthesis of hydrogels with specific shapes as the solution rapidly changes to gel phase as soon as it encounters the acid solution. Therefore, the frozen solution is used for the uniform gelation of the CMC solution. The frozen solution was then placed in the acid solution, which leads to the formation of CMC hydrogel. All hydrogels were readily washed in excess of D.I. water to remove unwanted acid residues.

3.3 Synthesis of magnetic nanoparticles

Magnetic nanoparticles were used in the formation of liquid 3D printed magnetic actuators. Magnetic nanoparticles were synthesized by the following procedure. Briefly, $FeCl_3$ (0.81g, 5mmol) and $FeCl_2 \cdot 4H_2O$ (0.5g, 2.5mmol) were dissolved in 10mL DD water in a round bottle flask and sealed by rubber septa cap. The solution was then sparged by nitrogen gas for 20mins to remove air completely. In another flask, 20mL 2M ammonia aqueous solution was degassed by the same way, and stirred at 750rpm high stirring rate at room temperature. Then the iron salts solution was transferred into ammonia solution by a 10mL syringe under nitrogen protection. After 1h, the reaction was stopped and black solids were collected by a magnet bar, which was washed with water repeatedly to remove ammonia and salts complexes.

3.4 FT-IR studies

All samples characterized for IR spectra were in dry state and were freeze dried prior to any further testing. FTIR–attenuated total reflection (ATR) was carried out on a Nicolet iS10 spectrometer, and 32 scans per sample were used. Briefly, first freeze-dried CMC hydrogel was analyzed for IR spectra. The hydrogel was then dissolved using 1 M NaOH solution and was dialyzed for 3 days with water change after every 3 hours followed by freeze drying. The dried sample was run for IR spectra.

3.5 ^{1}H NMR studies

^{1}H NMR studies were conducted on Avance 300 spectrometer. Samples were prepared by dissolving CMC in D_2O at 10mg/ml concentration. The ^{1}H NMR spectrum of CMC was carried out at 300 MHz.

3.6 Rheology test

Rheology tests were performed on a TA DISCOVERY HR1 hybrid rheometer, with a parallel plate (8 mm diameter, peltier plate steel). For investigating the gelation mechanism, 0.5% strain was used with angular frequency at 10.0 rad/s for time interval of 1500 seconds. Briefly, 100 µl CMC solution was placed in the plate and was subsequently subjected to 20 µl of 0.1 M HCl and 1 M NaOH to investigate the sol-gel phase transition. In another rheology tests, 0.5% strain was used with angular frequency ranging from 0.1 to 100 rad/s. 5 points per decade were analyzed for these tests. In these experiments, hydrogels were made prior to their rheology characterization.

3.7 Mechanical tests

The tensile characteristics of all the hydrogels was tested using the computerized electronic universal testing machine (WDW-02, SHIWI Instruments Inc., China), equipped with a 200 N load cell. All the tests were performed at a constant run speed of 10 mm/min. Sample preparation for tensile tests was carried by following the above-mentioned protocol for synthesising hydrogels with three hydrogels tested for the same concentration with dimensions, width ~ 7.5 mm , height ~ 3mm .

3.8 Swelling behavior of hydrogels

For measuring the water uptake capacity of the hydrogels, swelling ratios were calculated by immersing hydrogels in water and measuring changes in the mass at specific intervals. Three hydrogels for each group were designed and mean average value was used to plot the swelling ratio calculations. Each hydrogel was free dried for 24 hours prior to conducting swelling behavior tests. weight of hydrogels was calculated before swelling tests. Hydrogels were placed in the water and were taken out at predefined time intervals for calculating the changes in weight. Before calculating the mass of the hydrogels, surface tapping with filter paper was performed to remove the surface water from the hydrogels. Swelling ratio (Q) was calculated using the following equation[23],

$$Q = \frac{(w_s - w_d)}{w_d}$$

Where, w_s is the weight of the hydrogel in swollen state and w_d is the weight of the hydrogel in dry state.

3.9 Dissolution behavior of hydrogels

Three hydrogels were prepared in each group for all the concentration and mean average value was used to calculate the dissolution rates. To study the dissolution rates of the hydrogels, each hydrogel was thoroughly washed with the DD water prior to the tests. Dissolution degree was calculated in different pH buffer solutions of 7,7.5,8,8.5,9 which were prepared using tris solution and HCl solution. 3 samples of hydrogels were characterized for each pH groups and average was used to calculate the dissolution rates. Hydrogels were taken out after pre-determined time and were surface dried with the help of filter paper. Hydrogels were then weighed for change in the mass in response to the pH. Residual weight degree (W), was calculated using the following equation and was plotted against the time,

$$W = \frac{w_t}{w_i}$$

Where, w_i stands for the initial weight of the hydrogel and w_t for the weight of the hydrogel at time *t*.

3.10 Formation of Shape memory based paper origamis

For preparing the shape memory origamis, Whatman filter paper 4 was used as the source material of substrates for making different paper origamis. Initially, a 2*2 square piece of paper was cut from the filter paper and was spin coated with CMC solution on one side by covering the other side by applying wax. One side was covered with hydrophobic coating to allow the uniform coating of solution on just one surface. the paper was kept drying in room temp to remove excess water content. The origamis thus prepared were used for shape memory tests.

3.11 Preparation of multi-membrane hydrogels

For synthesis of multi membrane hydrogels, CMC stock solution was used as prepared earlier. First, a template or the core of the hydrogel system was made by pouring CMC solution in the small mold and was kept at -15°C for half an hour. 1 M HCl solution was then added to the frozen CMC solution and was kept for another half an hour at room temperature. After the formation of first membrane, the hydrogel was washed with excess of D.I. water to remove excess acid solution and was surface dried with filter paper to remove water for the formation of another membrane. The hydrogel was then dipped in the stock solution of CMC to form uniform layer of chitosan solution on the hydrogel which was then subsequently placed in 1 M HCl solution for 15 minutes for formation another layer of hydrogel. The hydrogel thus prepared was then washed with D.I. water to remove excess acid from the surface of the hydrogel before adding another layer on the hydrogel system. The above process was repeated for the number of layers as per requirement that need to be deposited. Prepared multi-membrane hydrogel was then washed with excess of D.I. water to remove excess acidic solution.

3.12 Chitosan as a 3D printing material

CMC was used as a 3D printing material by preparing a solution of 20% (w/v) solution. CMC solution was prepared by continuously adding chitosan into the solution under stirring at 60 C for 24 hours to achieve homogenous solution. CMC was added slowly to avoid the formation of lumps in the solution. Pressure used for CMC solution was 60 psi and needle size used was of 1 mm in diameter with glass plate as a substrate for printing. After printing the CMC on the glass substrate, the substrate was dipped into 1 M HCl bath for 1-2 minutes to crosslink the printed pattern. The

3D printed patterns were then thoroughly washed with the D.I. water prior to any further applications.

Using the same parameters for 3D printing, CMC solution was also used for liquid printing, in which spring shaped structures and threads were prepared by printing chitosan solution in the acid solution. For preparation of spring like structures, the needle was placed close to the bottom of the beaker containing 0.1 M HCl solution which allowed the rolling of the printing chitosan structures in the spring shaped patterns. The above prepared magnetic nanoparticles were also added in the CMC solution for the formation of magnetic responsive actuators. The above printed structures were also washed with D.I. water prior to any further experimental protocols.

3.13 Chitosan-CNT conductive patterns

For the synthesis of e-skin based biosensors and their potential applications, MWCNT was added in the chitosan solution and was homogenously mixed before using it as a bioink for 3D printing. First, MWCNT solution was prepared by mixing MWCNT (20 mg/ml) in the Pluronic F-127 solution (20 mg/ml) via ultrasonication for one hour. The CNT solution was then mixed with the chitosan solution in 2:1 (w/w) and was mixed for 15 minutes to achieve a homogenous solution. The as prepared CMC-CNT solution was then printed using the same printing parameters which were used for the chitosan 3D printing. The crosslinking procedure of the patterns used was also similar to the chitosan 3D printing by using 1 M HCl.

The printed conductive pattern was then used for designing e-skin based biosensor. Briefly, 3D printed pattern with dimension 1.5 X 1.5 cm^2 was connected on the opposite ends with the conductive copper (Cu) tapes making it a circuit which can conduct electricity if connected to the power source. The wire connected pattern was then covered by PDMS sheets to provide the

stability while in movement. The above described design was then used for sensor application when connected to the wireless sensor which was based on the wireless sensor for changes in resistance.

3.14 Microchannel systems

For designing the microchannel systems inside the polymer material, CMC was used as a material to make the patterns. Briefly, CMC was 3D printed in the patterns as per the requirements for the channels to be made in the PDMS sheet. The above-mentioned protocol for the preparation of 3D printing was followed for making the patterns. The 3D printed patterns were then used as a template around which the other polymer was prepared in such a way that chitosan pattern was entrapped inside the polymer material. For this procedure, CMC pattern was placed on the glass substrate which was then dipped in the PDMS pre-mix. Pattern containing PDMS sheet was then heated at 60 C for 1 hour to cure the PDMS with microchannel patterns. The PDMS sheet with chitosan pattern was then placed in the basic solution for 1-2 days depending on the complexity of the pattern to dissolve. The final prepared patterned PDMS sheet was then washed with excess of D.I. water to remove the excess solutions.

3.15 Controlled release studies

Three hydrogels were prepared in each group. To study the controlled release from the hydrogels, each hydrogel was thoroughly washed with the DD water prior to the tests. For the hydrogel systems containing fluorescein, following procedure was used. Briefly, 1 ml chitosan solution (30mg/ml) was mixed with 100 ul fluorescein solution (1mg/ml) and was then converted to gel phase in the presence of acidic solution to synthesize hydrogel with fluorescein entrapped inside

the gel. In case of multimembrane hydrogels, hydrogel layers were deposited on the core hydrogel made of fluorescein and CMC solution. Fluorescein containing hydrogel system were then washed with D.I. water to remove excess acid and fluorescein. To study control release behavior of the hydrogels, hydrogel systems were placed in D.I. water and other pH buffer solutions. After regular time intervals, 1 ml solution was taken out from the hydrogel system containing solution and was replaced by another 1 ml of the same pH solution to maintain the constant volume through the experiment. The samples were then tested in UV/Vis spectrophotometer (Ultrospec 4300 pro) for fluorescein released from the hydrogel.

Following formula was derived for calculating the release mass,

$y = 0.0868x + 0.0258$ with $R^2 = 0.9967$ for water and acidic solutions and

$y = 0.1889x + 0.0779$ with $R^2 = 0.9959$ for basic solutions

where y is the absorbance from uv-vis spectra and x is the concentration of fluorescein.

For calculating the release profiles, following equation was used,

$$m_l = m_i - (m_a + m_w)$$

Where m_l is the mass of fluorescein loaded in the hydrogel, m_i is the initial mass of fluorescein mixed with solution, m_a is the mass of fluorescein washed away during acid treatment and m_w is the mass of fluorescein washed away during washing with water.

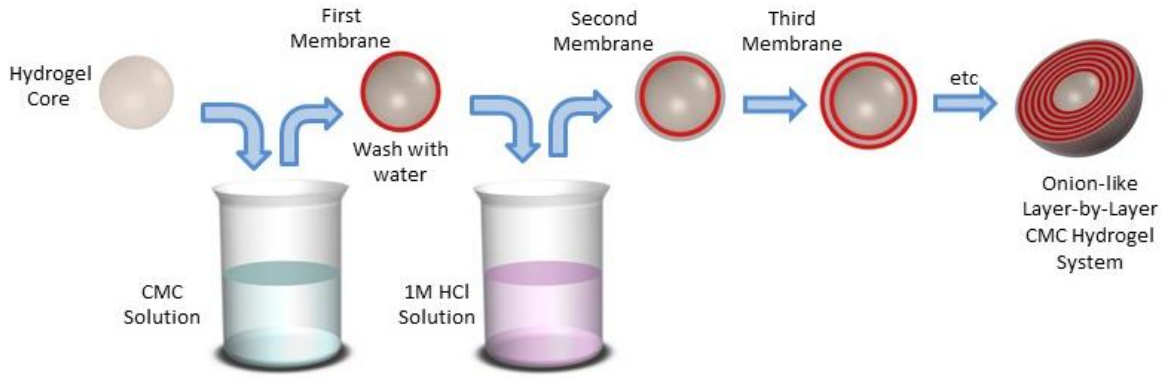
3.16 Amine groups detection

To determine the amount of amine groups in CMC, 1ml of different concentrations (1,2,3 and 5 w/v %) solutions of CMC was added with 0.2 ml ninhydrin solution (2% w/v). The samples were then tested in UV/Vis spectrophotometer (Ultrospec 4300 pro). All the tests were conducted in triplets.

Chapter 4: Results and Discussion

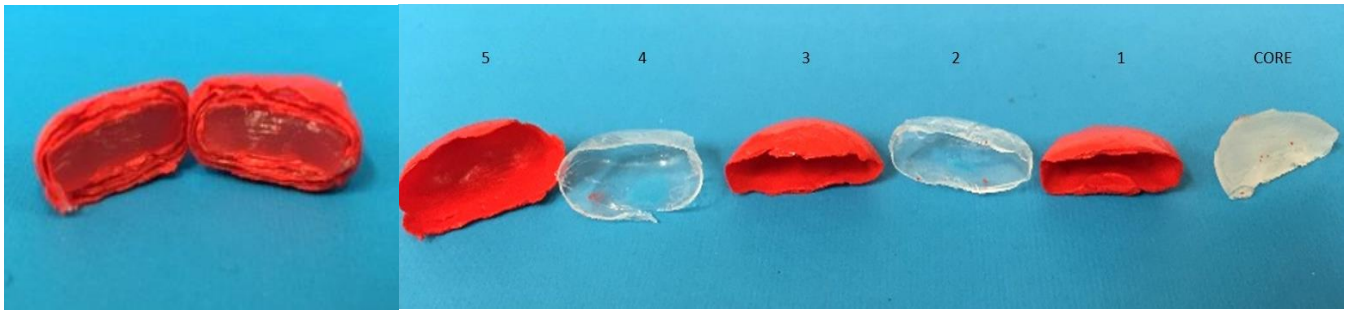
4.1 Layer by layer synthesis of multi-membrane hydrogels

Onion inspired hydrogels have multiple layers on one top of another and adds up to make a multi-membrane structure (Fig. 2a). In this work, we prepared a multi-membrane hydrogel system with 'n' (n=1, 2, 3, 4, 5, etc.) the number of layers depending on how many times the hydrogel systems were dipped in CMC and acidic solution (Fig. 2). Formation of each layer was a rapid process with time period of approximately 2 minutes taken for the formation of one layer.



(a)

(b)



(c)

(d)

(e)

(f)

Fig. 2. a) Schematic illustration of multi-membrane hydrogel synthesis. b) side-view shot of the multimembrane hydrogel with peeled layers. c) Cross-section of the multi-membrane hydrogel and layer by layer extraction of all the layers from single multi membrane hydrogel. d) control hydrogel shells. e) fluorescein mixed hydrogel shells under UV light. f) Magnetic nanoparticles laden gel shell as magnetic actuator.

Briefly, CMC hydrogel core was first made by freezing the CMC solution to maintain the structural integrity and to allow the slow phase transition. Then, the frozen CMC was dipped in acid solution and it was thereby used as a template to make different hydrogel layers around it, thus producing desired multi layered structure. Each layer of the hydrogel was synthesized on the previous layer same as onion structure and also could be peeled off thereby completely mimicking onion. As

shown in Fig.1c, an onion like hydrogel with 5 layers was made. Each layer was then peeled off. Orange color was added to every successive layer after clear hydrogel layer. It can be seen in the Fig. 1c, hydrogel layers are peeled off and arranged with different peeled layers. The structural integrity of the hydrogel layers was still intact and could maintain its original hemispheric shape (Fig. 2c and d).

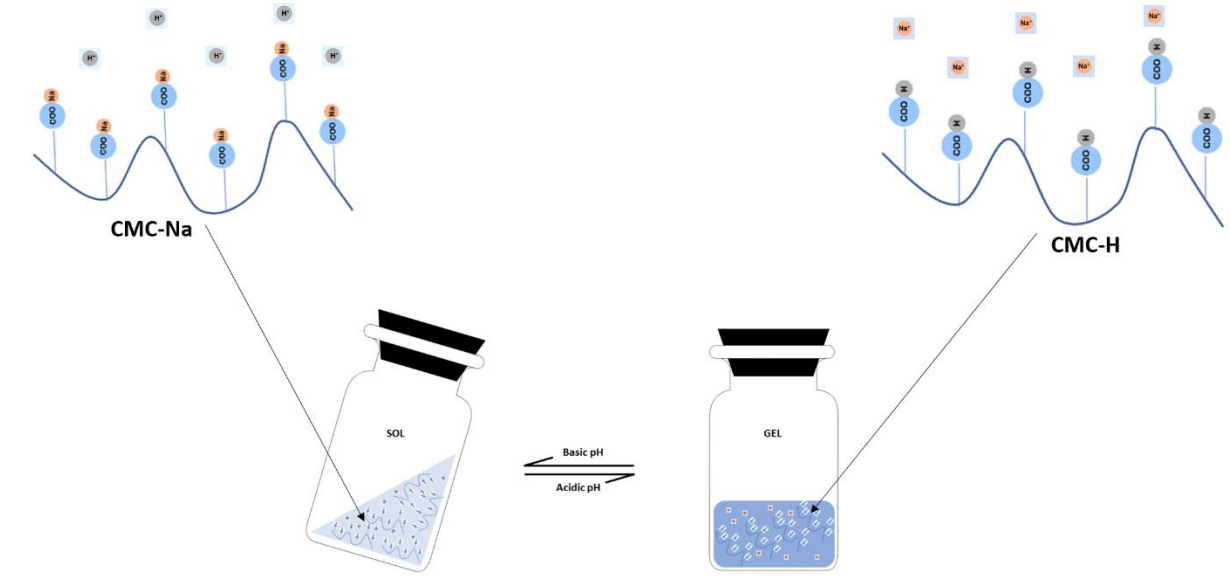
To further demonstrate peeled membrane's structural attributes, each membrane was loaded with fluorescein. Structural integrity of the peeled membranes was found be intact as they were observed to be freely swimming in solution mimicking the fluorescent jellyfish-like structures (Fig. 2e). Guided motion of the peel able layers was achieved by adding magnetic nanoparticles in the hydrogel layers (Fig. 2f). Thereby, because of the structural integrity of single hydrogel layers and versatility of able to inculcate different materials, these layers can act as an actuator with potential applications in biomaterials based robotic systems.

4.2 Structure and mechanism

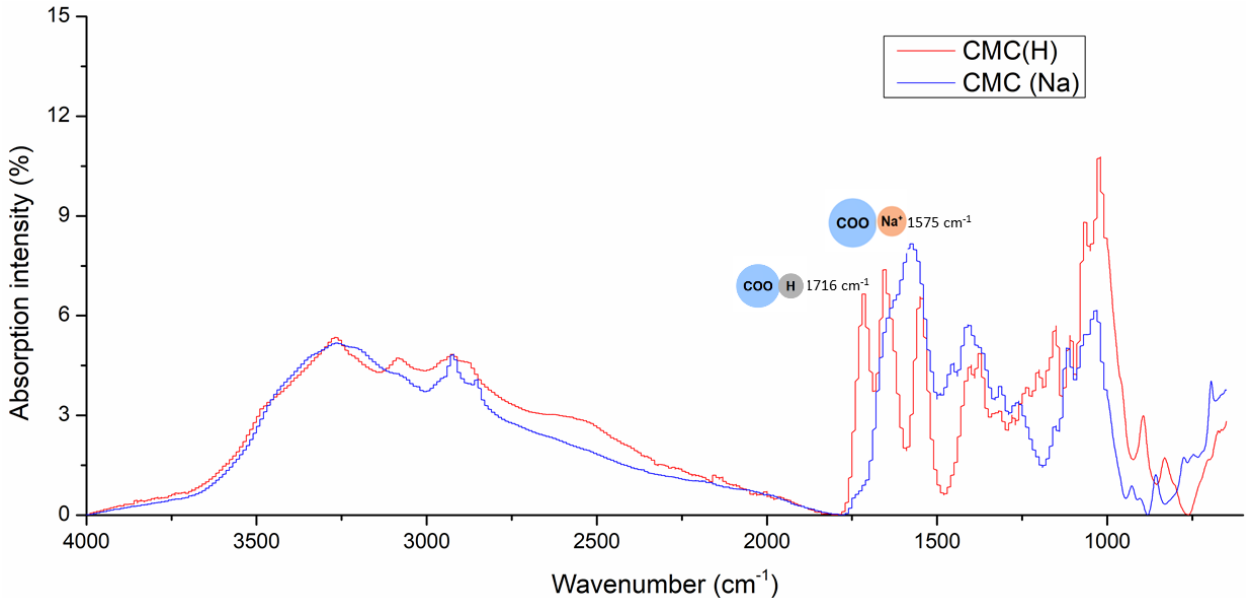
The CMC structure and site of carboxymethyl substitution was identified by using FTIR spectra. The FT-IR spectra of freeze dried carboxymethyl chitosan (CMC-H) during the gel phase and freeze dried carboxymethyl chitosan-sodium salt (CMC-Na) after the dissolution in sodium hydroxide solution are shown in Fig. 3b. IR spectra for all the chitosan derivates show peaks at range 3100-3300 cm^{-1}, which can be ascribed to the O-H stretching (OH of hydroxyl groups, OH of COOH), also peaks at 2925-2950 cm^{-1} are ascribed to the C-H stretching [110]. The peaks at 1575 cm^{-1} and 1432.11 cm^{-1} can be attributed to the respective asymmetric and symmetric stretch vibrations of -COO-Na [241]. It can also be observed that, the peak for NH_2 was not observed in the spectrum, meanwhile the peaks for OH group can be observed at 1027.4 cm^{-1} which indicates

the characteristics of N-carboxymethyl chitosan sodium salt [67, 110, 242]. To further confirm the position of substitution, ninhydrin assay test was conducted to determine the presence of NH_2 groups in the CMC. It has been reported that, the reaction of ninhydrin with amine groups (NH_2) leads to the chromophore formation of Ruhemann's purple (RP) (λmax 570 nm)[243]. Lysine-ninhydrin was used to plot the standard equation for the amine concentration. Different concentrations of CMC (0.5,1,3 and 5% w/v) was tested against ninhydrin solution for the presence of amine groups which resulted in negative results with no color change in the solutions. It can be observed from fig 3d, the absorption peak for CMC-ninhydrin forming RP (λmax 570 nm) cannot be detected, which can be the result of very less reaction of ninhydrin with CMC. Therefore, it can be confirmed that the CMC have substitution on the N site, which is in compliance with the seller's details of N-carboxymethyl chitosan.

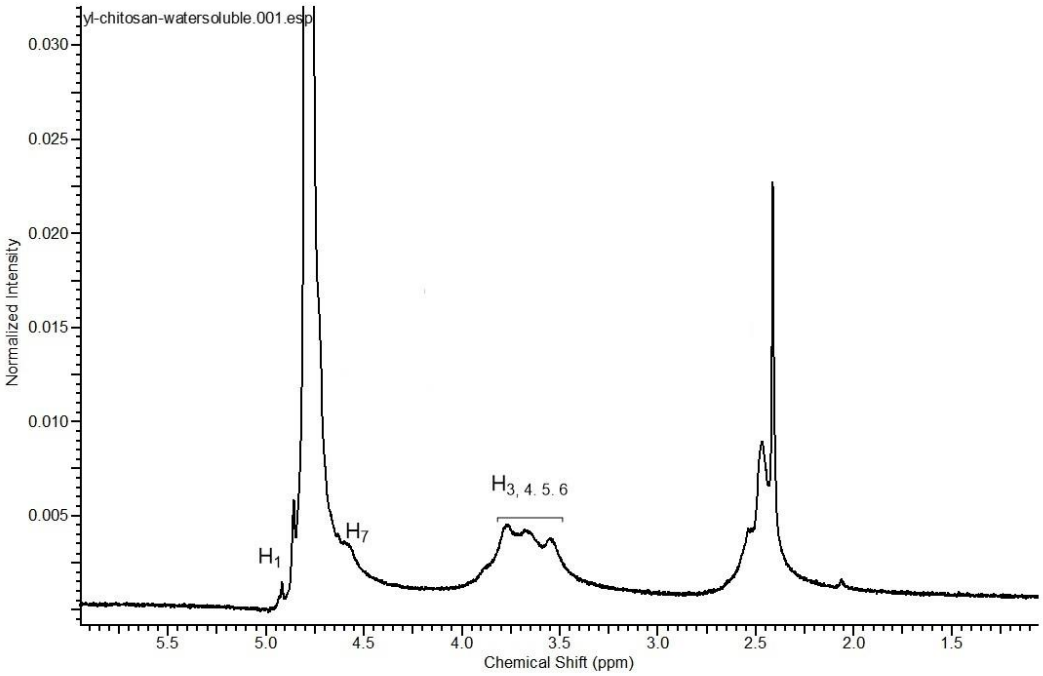
FT-IR characterization was also used to quantify the ionic association and disassociation of carboxylic group derivate in response to the protonation and deprotonation of the CMC caused by the changes in pH values. After addition of acid in the CMC-Na solution, formation of CMC-H leads to the transition to gel phase which was then repeatedly washed with DD water and freeze dried for FTIR analysis. IR spectra of the carboxymethyl chitosan (CMC-H) shows the apparition of peak at 1716 cm^{-1} which is attributed to carboxyl acid group, drifted to the left as compared to carboxyl peak for sodium salt derivative because of the formation of -COO-H after treatment with acidic solutions. The stretching of peak at 1065 $cm^{-1}$for CMC-H can be ascribed to the C-O stretching of the carboxymethyl chitosan[67]. When the hydrogel was added to the basic solution, the FT-IR spectrum of the resulting dissolved gel shows the ascent of peak at 1575 cm^{-1}which can be attributed to the formation of carboxymethyl chitosan- sodium salt derivative, disappearing the peak at 1710 cm^{-1} for -COO-H groups.


CMC-Na
CMC-H
SOL
GEL
Basic pH
Acidic pH

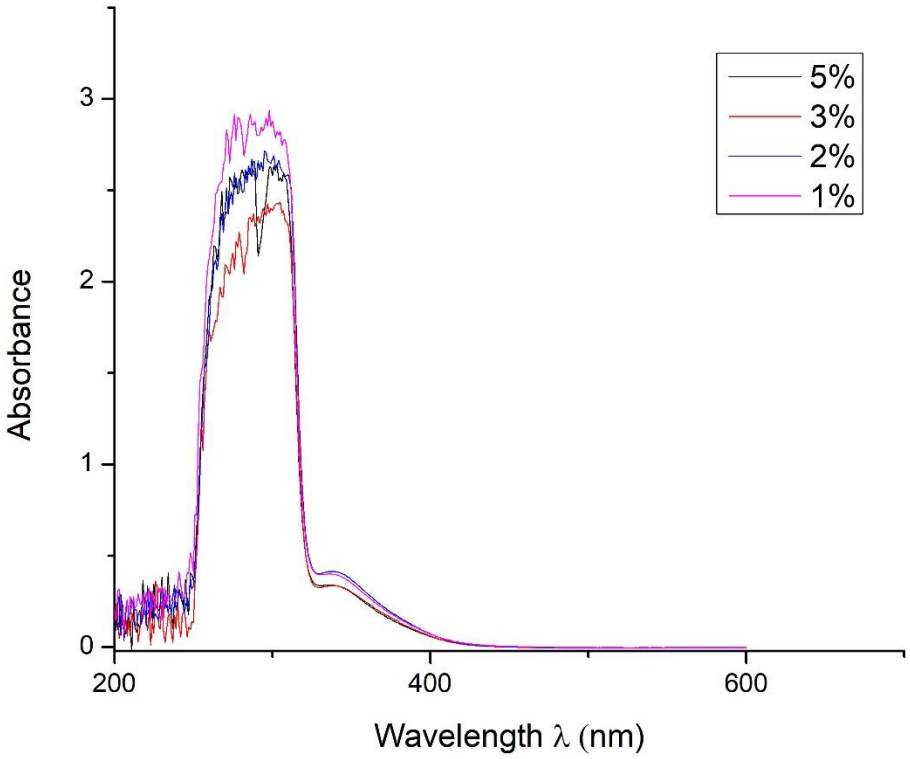
(a)


CMC(H)
CMC (Na)
COO Na+ 1575 cm-1
COO H 1716 cm-1
Absorption intensity (%)
Wavenumber (cm-1)
0
3
6
9
12
15
4000
3500
3000
2500
2000
1500
1000

(b)


yl-chitosan-watersoluble.001.esp
Normalized Intensity
0.030
0.025
0.020
0.015
0.010
0.005
H1
H7
H3, 4, 5, 6
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
Chemical Shift (ppm)

(c)


5%
3%
2%
1%
Absorbance
3
2
1
0
200
400
600
Wavelength λ (nm)

(d)

Fig. 3 a) Schematic illustration of the mechanism involved in sol-gel transition of CMC. b) FT-IR spectra of carboxymethyl chitosan-sodium salt (CMC-Na) and carboxymethyl chitosan (CMC-H) during the gel phase. c) H-NMR spectrum of CMC. d) uv-vis spectra of CMC-ninhydrin solution (1,2,3,5 % (w/v)).

The ^{1}H NMR spectrum of CMC at 300 MHz is carried out in D_2O. The signal at 4.85ppm corresponds to proton H_1 of chitosan. The signals in the range of 3.40-3.90 ppm are ascribed to protons H_3-H_6. The signal at 4.45-4.55 ppm is attributed to protons H_7 (CH_2) of carboxymethyl group. Thus ^{1}H NMR data confirm that we have the N-carboxymethyl chitosan sodium salt compound.

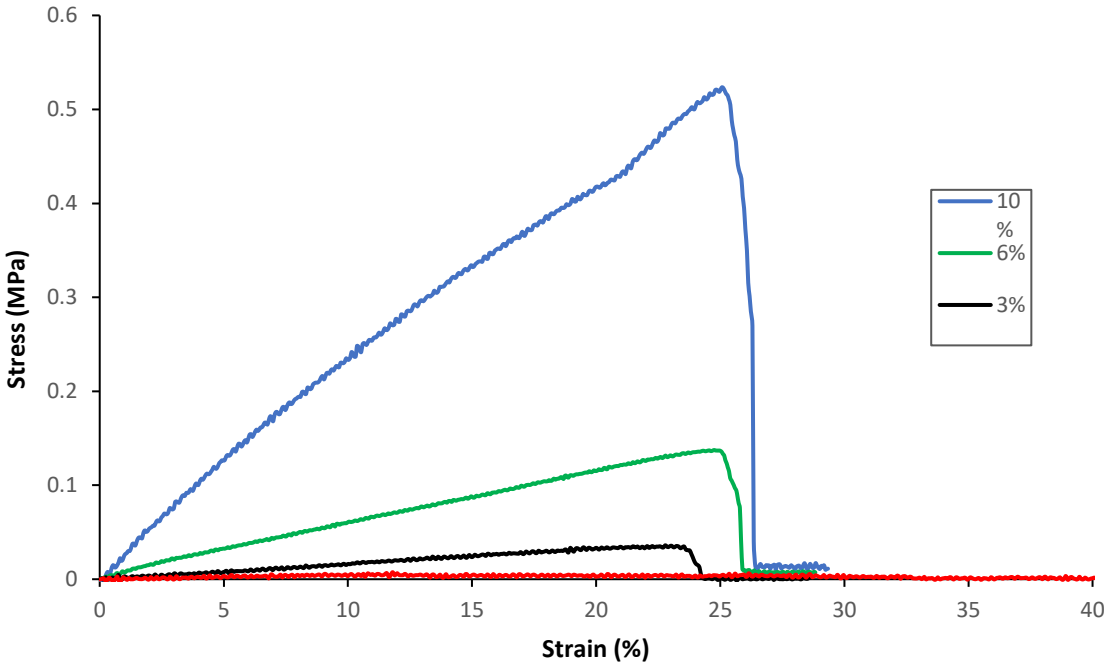
The gelation of CMC solution can be credited to the two processes (Fig. 3a), (i) ionic dissociation between the carboxymethyl chitosan-sodium salt (-COO-Na) and water molecules leading to solubility change and (ii) simultaneously formation of carboxylic acid group (CMC-H). The addition of HCl favors the displacement of sodium ions due to the ionic interaction between sodium ions and chloride ions that form the sodium chloride on one hand, and the carboxylic acid one another hand (as shown in the FTIR spectra, Fig 3b). When the HCl solution is added to the CMC-Na water solution, there is an increase in the proton (H^+) concentration leading to protonation of (-COO^-) and formation of (-COOH) which leads to reduced solubility, which was confirmed by FT-IR analysis. Formation of carboxylic acid, directs to low solubility of the chitosan solution because of the less solubility of carboxyl groups in water, thus initiating the gel phase conversion of the CMC solution. Also, the interaction of CMC with water molecules brings an increase in the polymer chain mobility in the water solution. As a result of high chain mobility and lower polymer dissolution, gel phase of the carboxymethyl tends to have entrapped water inside the gel phase thus providing a hydrogel like structure instead of aggregation. However, when NaOH solution is added to the gel phase of CMC, there is a phase transition to sol phase caused

by the increase in the number of hydroxide ions (OH^-) in the solution leading to the ionic dissociation of (-COOH) and formation of (-COO-Na^+).

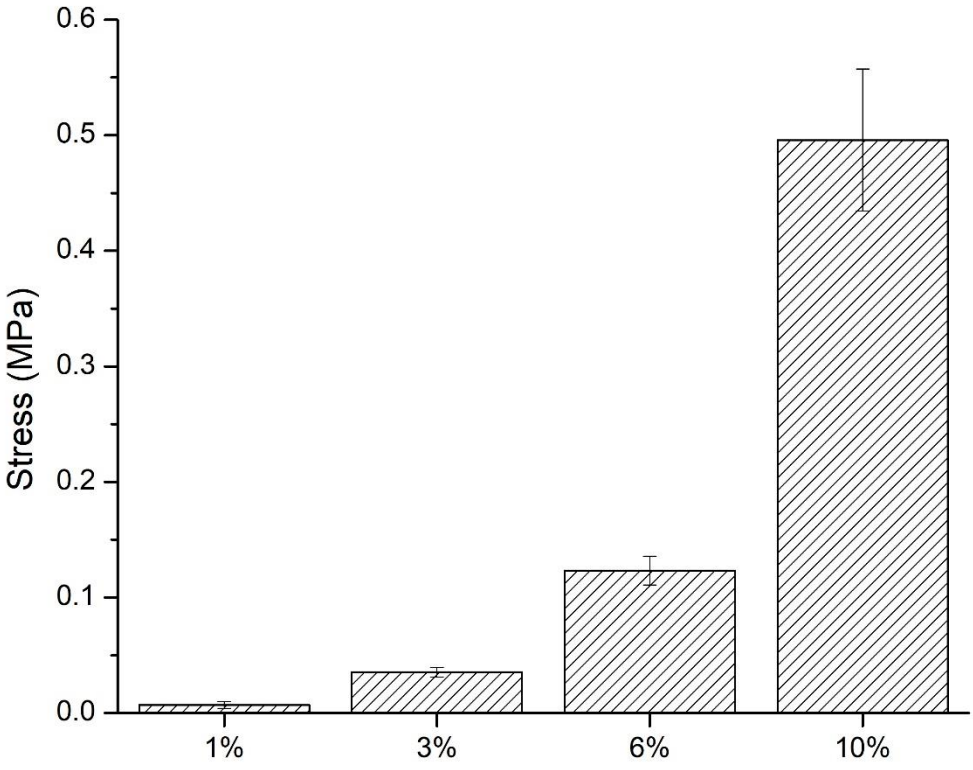
The pH based sol-gel conversion is a reversible process as carboxylate group can be protonated and deprotonated by changing the pH of the solution, thereby controlling the solubility of the CMC solution.

4.3 Mechanical Properties of carboxymethyl chitosan hydrogels

To calculate the mechincal properties of the CMC hydrogels, the tensile strength of the hydrogels was calculated and compared. It was observed from the tensile strength graphs in fig. 4a, that the mechanical strengths of the CMC hydrogels increased with increasing the concentration of CMC solution in the hydrogel. Hydrogels with lowest concentration of CMC solution (1%), exhibited the lowest tensile strength (0.01 MPa) as compare to the hydrogels formed by using 10% CMC solution (0.226 MPa). The hydrogels made from intermediate concentrations (3% and 6%), had the tensile strength in the region of 0.03 MPa and 0.1 MPa respectively. The increase in the tensile strength of the hydrogels with increasing the concentration of solution can be attributed to the availability of more carboxymethyl groups of CMCs available to participate in the phase transition, which increases the density and strength of gel phase.


Stress (MPa)
Strain (%)
10 %
6%
3%
0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
25
30
35
40

(a)


Stress (MPa)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
1%
3%
6%
10%

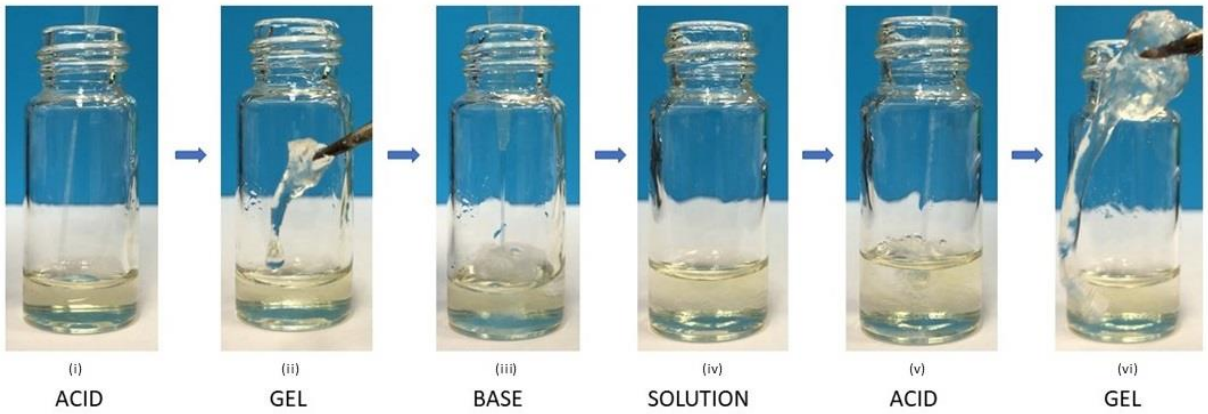
(b)

Fig. 4 Mechanical properties of CMC hydrogels. a) Tensile stress-strain curves of CMC hydrogels with different concentration (1, 3, 6, 10%). b) column bar with error bars for the tensile strength of CMC hydrogels with conc. 1%, 3%, 6%, 10%

4.4 Sol-gel behavior of carboxymethyl chitosan

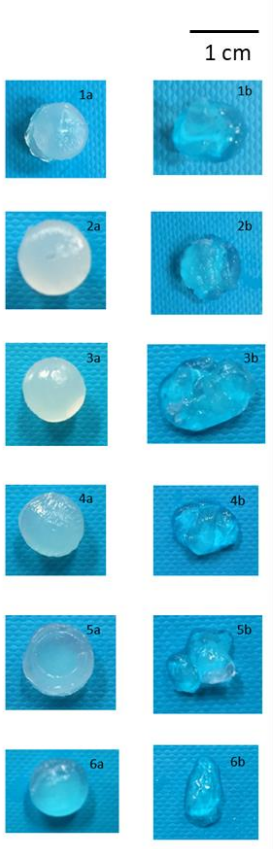
To demonstrate the sol-gel conversion of CMC solution (fig. 5a), acidic solution (pH 1) was added to the CMC solution (10%). It was observed that after the addition of acidic solution, a quick phase change takes place converting to gel phase in few seconds. The formed hydrogel can be again converted back to the sol by changing the pH to basic scale of 10. The sol-gel conversion of CMC was a reversible process and was again converted back to gel-phase by changing the pH to 1. In order to further clarify the sol-gel transition with different concentrations of CMC and varied pH values, we designed the experiments in which we first used different concentrations of CMC (1,2,3,4,5 & 6 w/v %) and different pH values (1,2,3,4,5 & 6) to identify the threshold concentration and pH value at which gelation initiates (Fig. 5b). It was observed that gelation was initiated for all the concentrations of CMC solutions at pH values below 3. Gel structure was more stiff and stable when gelation occurred at pH 1 as compare to the pH 2. It can hence be derived that, gelation threshold was somewhere between pH 2 and 3. In next step, CMC solution of 3 % was used for further study (Fig. 5c). The pH solutions ranging from 2.05, 2.2, 2.3, 2.4, 2.5, 2.6 and 2.9 were used to determine the threshold pH value for gelation. It was observed as the pH started to increase, the morphology of the gels formed was less stiff and stable. At pH 2.5, only fragments of CMC were found in the solution which were settled down at the bottom as the precipitates. Further increasing the pH didn't lead to any gelation and CMC was present in solution phase only. Therefore, it was hence demonstrated that critical pH value for gelation is 2.5. To verify that

critical gelation pH is same for different concentration of CMC solution, 1% and 5% CMC solution was tested for the phase transition at pH 2.5 and exhibited the formation of hydrogel in both cases.



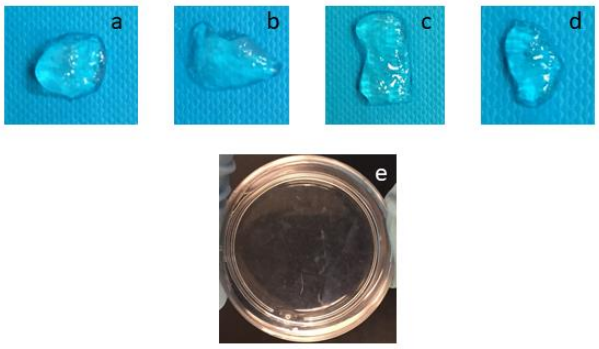
(a)

Sample	Chitosan (w/v %)	pH	Gelation
1a	1	1	+
1b	1	2	+
1c	1	3	-
1d	1	4	-
1e	1	5	-
1f	1	6	-
2a	2	1	+
2b	2	2	+
2c	2	3	-
2d	2	4	-
2e	2	5	-
2f	2	6	-
3a	3	1	+
3b	3	2	+
3c	3	3	-
3d	3	4	-
3e	3	5	-
3f	3	6	-
4a	4	1	+
4b	4	2	+
4c	4	3	-
4d	4	4	-
4e	4	5	-
4f	4	6	-
5a	5	1	+
5b	5	2	+
5c	5	3	-
5d	5	4	-
5e	5	5	-
5f	5	6	-
6a	6	1	+
6b	6	2	+
6c	6	3	-
6d	6	4	-
6e	6	5	-
6f	6	6	-



(b)

Sample	Chitosan conc. (w/v%)	pH	gelation
a	3	2.05	+
b	3	2.2	+
c	3	2.3	+
d	3	2.4	+
e	3	2.5	+ (fragments in solution)
f	3	2.6	-
g	3	2.9	-

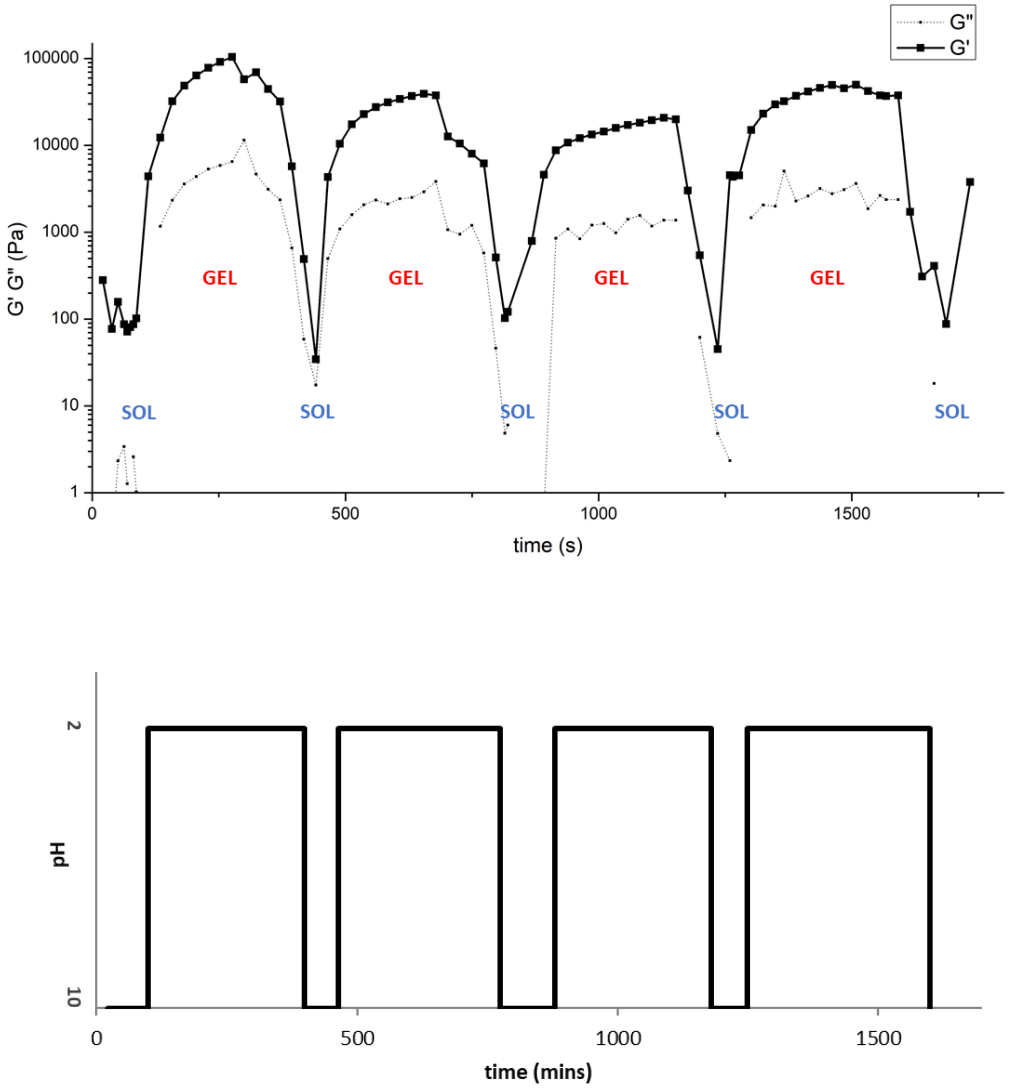


(c)

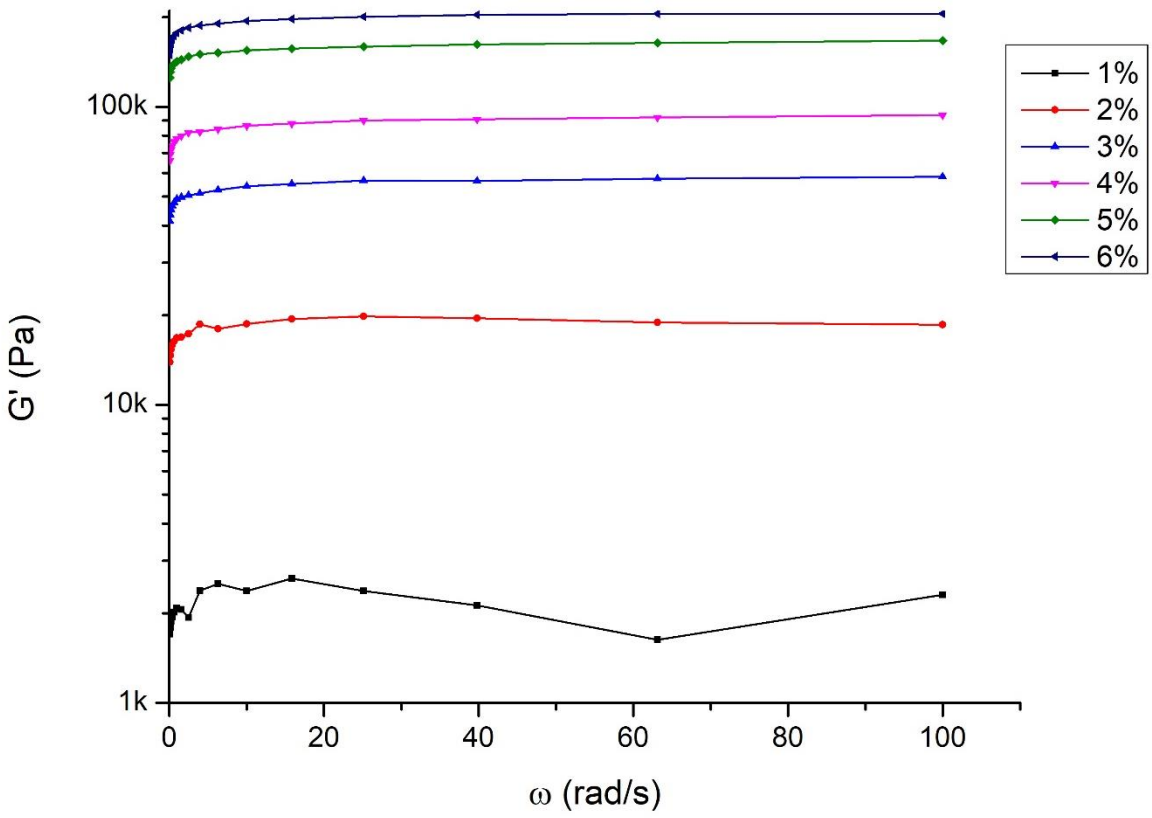
Fig. 5) a) Sol-gel conversion by acidic and basic transition. Determination of critical pH value and concentration of CMC for gelation; b) Different pH values and concentrations used for gelation and corresponding images of the gels formed; c) pH values between 2 and 3 for gelation and corresponding images of the gels formed ('+' sign indicating the gelation and '-' sign indicating the solution phase).

The gelation of CMC solution in an acidic pH is contrastingly different from the unmodified chitosan as acidic medium is most commonly used to dissolve chitosan because of higher pKa value of chitosan in the range of 5.6 – 6 [19, 20]. To further analyze the sol-gel conversion, storage modulus (G') and loss modulus (G") were calculated against the time interval for conversion by rheological studies (Fig. 6a). The rheology graph was calculated in a continuous reaction set up to plot real time changes in values of storage modulus and loss modulus as a response to change in pH values. According to the values of G' and G" in rheological graph (fig. 6a), the sol-gel phase transition of the CMC solution can be divided into three phases, solution, intermediate and gel phase[244]. During the solution phase of the CMC, the values of G' and G" were lower and in the region of 100 Pa. However, on addition of the acidic solution, the values of G' tends to escalate, which forms the intermediate phase. These intermediate points on the graph are observed because of the change in values of G' and G" during phase conversion of CMC solution. Once the CMC

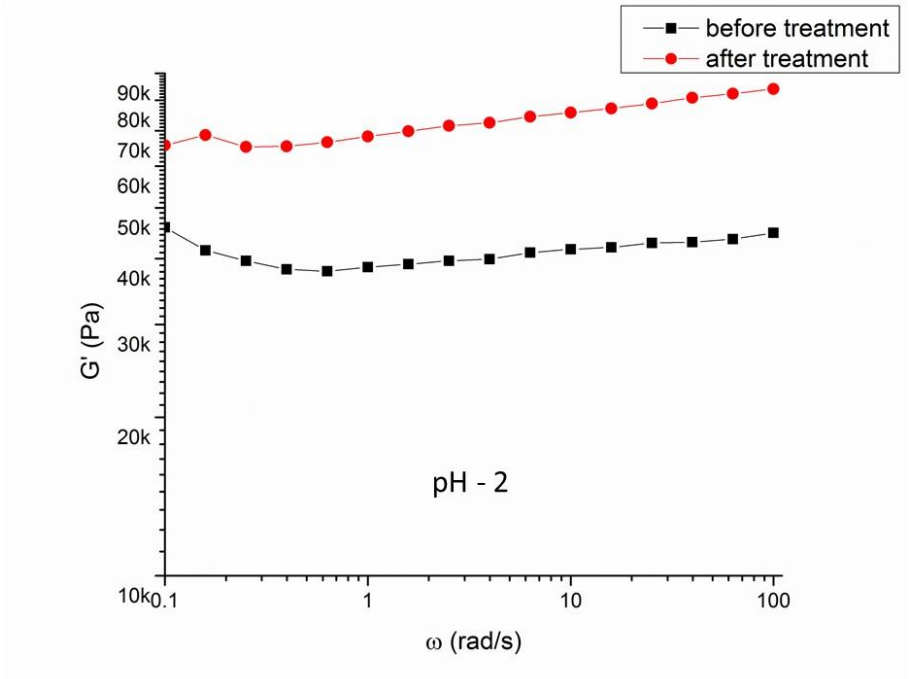
was converted to gel phase, the values of G' and G" reach relatively higher points in the region of 100000 Pa. The continuous conversion of sol-gel and gel-sol on changing the pH values causes changes in the values of storage modulus and loss modulus. This pH responsive sol-gel conversion properties open a whole new wide range of applications for these hydrogels-based systems.



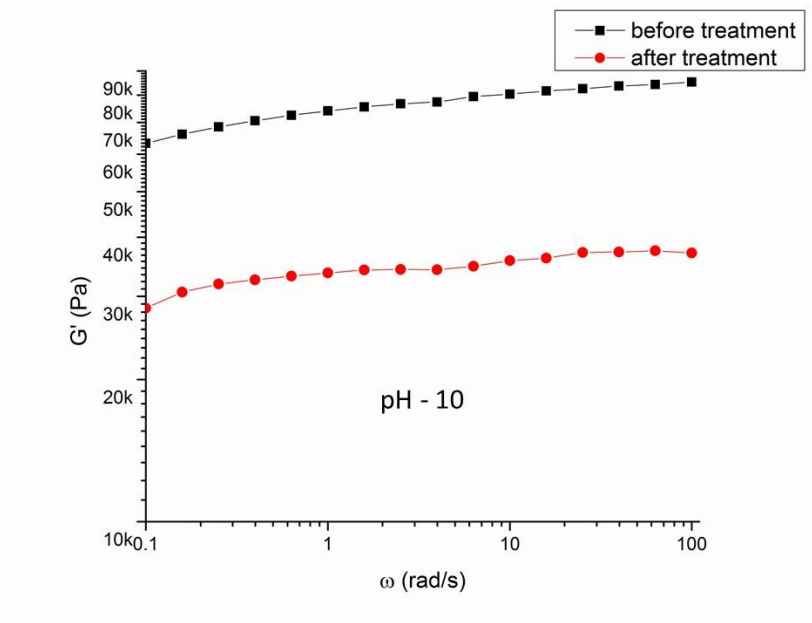
(a)


G' (Pa)
100k
10k
1k
0
20
40
60
80
100
ω (rad/s)
1%
2%
3%
4%
5%
6%

(b)


before treatment
after treatment
G' (Pa)
90k
80k
70k
60k
50k
40k
30k
20k
10k
0.1
1
10
100
ω (rad/s)
pH - 2

(c)



(d)

Fig. 6 a) Rheology graph of G' and G" with time change for gel-sol conversion. b) Rheology graphs for storage modulus of the chitosan hydrogel with different concentrations (1,2,3,4,5,6% w/v). c) Rheology graphs for storage modulus of the hydrogel before and after in acidic solution. d) Rheology graphs for storage modulus of the hydrogel before and after in basic solution.

Rheology was also used to monitor the effects of acid and base on the storage modulus of the hydrogels. Storage modulus was chosen here because it can be used to determine capability to maintain structure stability of the hydrogel [245]. Different concentrations of CMC solution (1,2,3,4,5 & 6 w/v %) in their gel phase was analyzed for the values of storage modulus to comprehend the difference in stiffness and the structural strength of the physical hydrogels (Fig. 6b). It was observed that storage modulus, G', for 1% chitosan hydrogel (2306 Pa at 100 ω (rad/s)) was low because of the fewer carboxylic groups available for gel phase transition due to less concentration. There was an evident increase in the values of the G' for the increasing concentrations of the CMC solutions (2%–18563 Pa, 3%–58262 Pa, 4%–93636 Pa, 5%–166539 Pa, 6%–205143 Pa at 100 ω (rad/s)). Increase in the values of storage modulus with increasing the

concentrations of CMC can be rationalized, as number of carboxylic groups responsible for the gel phase of the solution also increases with increase in the concentration. Therefore, it can be quantified from the rheology tests that with the increase in the concentration of the CMC, there is an increase in the stiffness of the hydrogels.

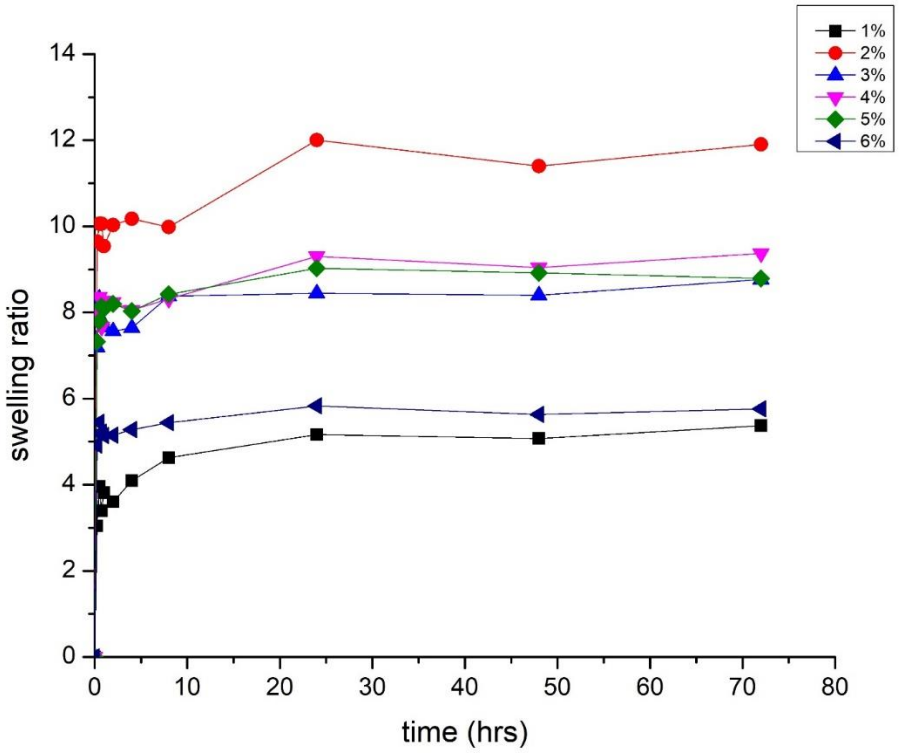
According to our hypothesis, hydrogel was expected to become more stable in acid solution thereby increasing the value of storage modulus. As expected, the value of storage modulus was increased for the CMC hydrogel (3%), when it was placed in acidic solution with pH value 2 for 1 hour (Fig. 6c). Presence of acidic solution leads to increase in the protonation of the CMC hydrogel and makes it stiffer and thereby increasing its strength with increase in the physical crosslinking of the gel phase. However, when hydrogel was placed in the basic solution of pH 10 for 30 minutes (Fig. 6d), the sol-gel conversion caused by the deprotonation of the carboxylic group changes the dissolvability of the chitosan which leads to the transformation from the gel phase to the solution phase. Gel-Sol transformation leads to the decrease in the strength of the hydrogel because of the increment in the solubility of the chitosan solution, converting it to liquid like gel phase with less strength as compared to the solid like gel phase. This change in the structural strength of the sol-gel phases is evident from the respective decrease and increase in the value of storage modulus which demonstrates the different structure satiability of the hydrogel in different pH solutions.

4.4 Swelling and dissolution behavior

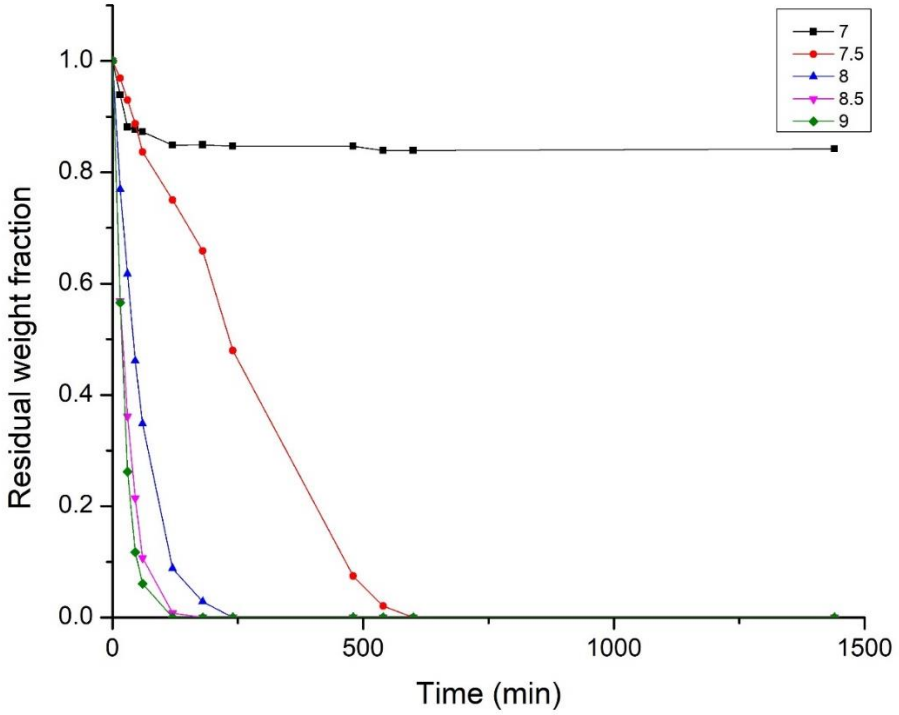
Hydrogels are often regarded as the materials with unique absorbing capabilities. Swelling tests were conducted to realize this property. Freeze-dried hydrogels were placed in the water solution and were measured for increase in the mass in response to the water uptake. It was observed that

(Fig 7a), hydrogels with high concentration (6%) and low concentration (1%) had the swelling capability of approximately 5 times the dry weight of the hydrogel. As compare to the other hydrogels (3,4,5 %), hydrogels with 2% concentration were observed to have higher swelling ratio. The low swelling behavior of 1% hydrogels can be attributed to the less number of carboxyl groups available for hydrogel formation therefore the hydrogel thus formed have weak interconnections and is not able to retain the water. Whereas, in case of 6% hydrogel, the water uptake is low because of the high amount of carboxyl groups present for hydrogel formation which leaves few spaces between the hydrogel for water uptake.

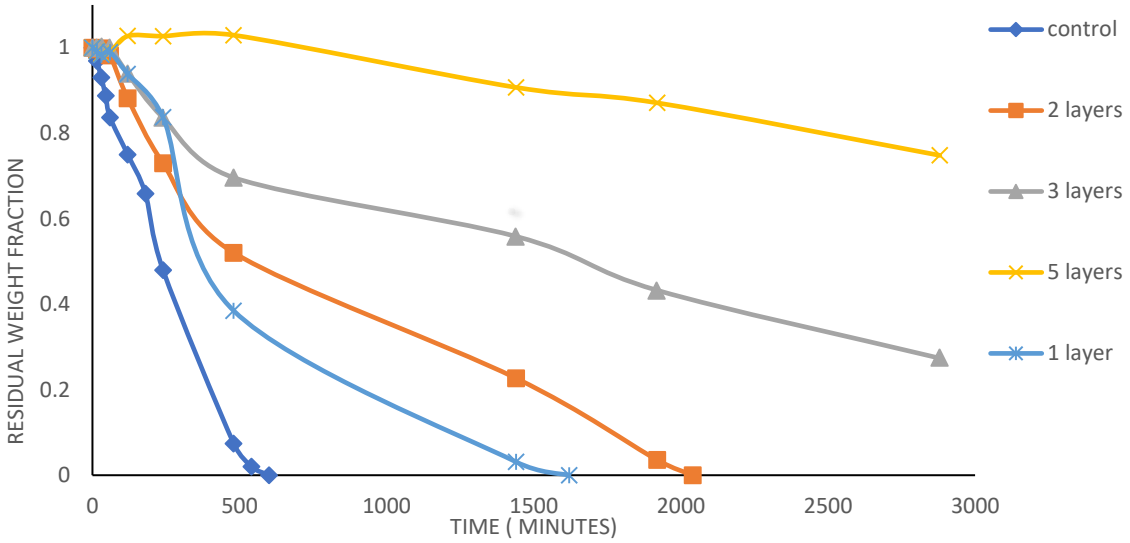
Different basic pH values have different effects on the CMC hydrogel and to determine the dissolution rates, chitosan hydrogels were studied for their change in weight in different pH values (7,7.5,8,8.5 & 9) [246]. It was observed that (Fig 7b), hydrogels were stable in pH 7 solution with not much change in their weight ratio and remained constant in the solution even until 3 weeks. Increasing to a basic pH value, the solutions was seen to have a direct effect of increase in the dissolution rate of the hydrogel. In pH 7.5 solution, hydrogel was reduced in weight to 14 % in 4 hours in comparison to the full dissolution of the hydrogel in pH 8 solution in 4 hours. Increasing the pH values further higher to 8.5 and 9, decreased the time of dissolution and hydrogels were completed reduced to 0% in 3 hours and 2 hours, respectively.


1%
2%
3%
4%
5%
6%
swelling ratio
time (hrs)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80

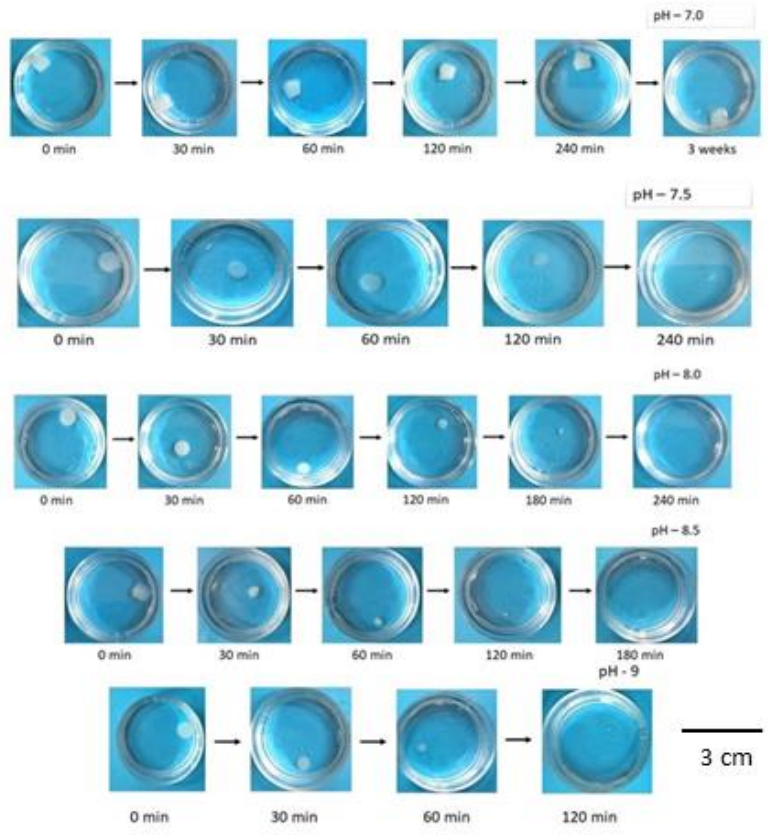
(a)


7
7.5
8
8.5
9
Residual weight fraction
Time (min)
0.0
0.2
0.4
0.6
0.8
1.0
0
500
1000
1500

(b)


RESIDUAL WEIGHT FRACTION
1
0.8
0.6
0.4
0.2
0
0
500
1000
1500
2000
2500
3000
TIME (MINUTES)
control
2 layers
3 layers
5 layers
1 layer

(c)


pH – 7.0
0 min
30 min
60 min
120 min
240 min
3 weeks
pH – 7.5
0 min
30 min
60 min
120 min
240 min
pH – 8.0
0 min
30 min
60 min
120 min
180 min
240 min
pH – 8.5
0 min
30 min
60 min
120 min
180 min
pH - 9
0 min
30 min
60 min
120 min
3 cm

(d)

Fig. 7 a) Swelling ratio of chitosan hydrogel in water. b) Dissolution rates of chitosan hydrogel in different basic pH solutions (7,7.5,8,8.5,9). c) Dissolution rates of multi membrane chitosan hydrogels with different number of layers (0,1,2,3,5). d) Images of hydrogels at different pHs for calculating dissolution rates.

Control over dissolution rates is the most prime objective for engineering a biomedical hydrogel system to be used in medicinal applications[34, 36]. It helps in the removal of hydrogel after it has achieved its full potential objective and for the synthesis of controlled release systems. We synthesized the multi membrane hydrogels which had different dissolution rates depending on the number of hydrogel layers. It can be observed from Fig.7c, there was a decrease in the dissolution time for the hydrogels with additional layer of hydrogel in pH 7.5 solution. Hydrogels with 5 membranes were still stable after 32 hours. Addition of 2 layers increased the stability of hydrogels by up to 24 hours as compared to the hydrogel with no membrane in the same pH solution. Therefore, with the help of multi membranes, we can design different type of hydrogels systems with different dissolution rates depending on the end user applications.

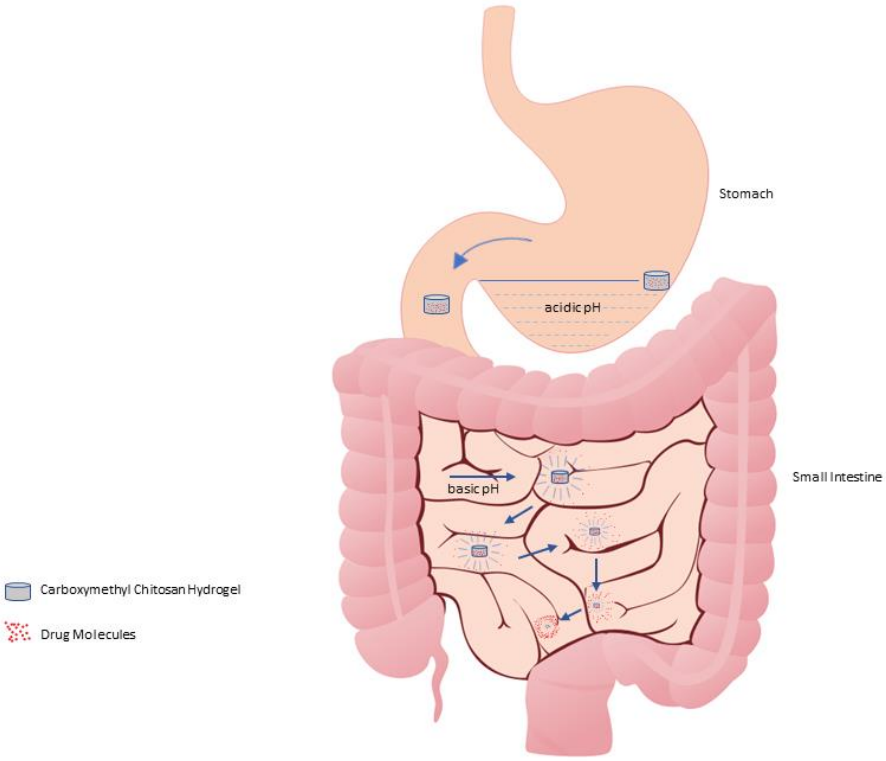
4.5 Cumulative controlled release

Intestine specific drug delivery systems are usually degraded or dissolved in the harsh acidic (pH 1.5-3.5) stomach environment before reaching the intestines (pH 7-7.5). The hydrogels have capability to sustain acidic environment and dissolve in basic conditions and can found potential applications as intestine-specific drug delivery models (fig. 8b). To demonstrate the potential drug release profiles, cumulative controlled release studies were conducted with a fluorescein release model which was analyzed with uv-vis spectroscopy. First, the pH specific release profiles from CMC hydrogels were conducted in two different pH conditions similar to that of stomach (1.5) and intestine (7.5). The hydrogel was made by homogenously mixing CMC solution with fluorescein for half an hour and was then washed with water to remove excess fluorescein. The

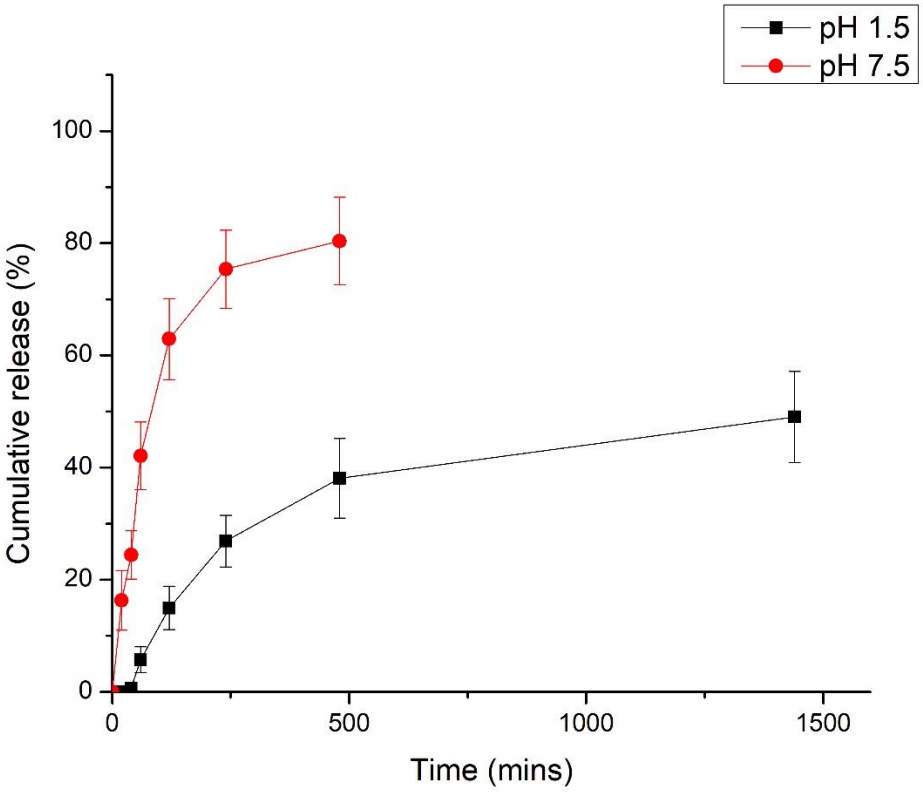
obtained results were plotted as percentage of cumulative fluorescein concentration released from hydrogel v/s time interval (fig. 8c). It was observed that release rates for CMC hydrogel (3%) was higher in basic pH conditions (80%) as compared to the acidic conditions (42%). The release studies are in accordance with the dissolution studies which states that CMC hydrogels can sustain their shape and size in acidic medium and gets dissolved in basic conditions thereby releasing drug molecules into the system. Different concentrations of CMC hydrogels have been found to have different effects on the physicochemical properties of the hydrogels. Further extending the cumulative release studies, we carried out two experiments to gain more insight on the release rates of the hydrogel. First, CMC hydrogels (1, 3, 5 % w/v) (Fig. 8d) laden with fluorescein were studied for the effects of CMC concentration on release studies; second, effect of multi-membrane on the release rates was studied for the hydrogel-fluorescein complex in pH 7.5 (intestine environment) (fig. 8e). The obtained results were again plotted as percentage of fluorescein concentration released from hydrogel v/s time interval (Fig. 8 d-e). Different release rates were witnessed for the different concentrations of the CMC hydrogel. Hydrogels with lower concentration (1%) had higher release rates as compare to the hydrogels with higher concentration (Fig. 8d). Release rates were observed to be comparatively lower for hydrogels with higher concentrations (5 & 3 %). Difference in the release rates from CMC hydrogels was found to be dependent on the concentration, with an evident decrease in the release profile for increasing concentrations of the CMC solution. The decrease in the release profile can be supported as increasing the concentration of the solution will increase the number of carboxylic groups in the solution, available for the formation of gel phase making it stiffer and tightly arranged structure, which was earlier explained by the rheology tests. The stiffer structure in higher concentration

hydrogels, allows slow diffusion of the materials from the gel phase which makes them slow release hydrogel systems.

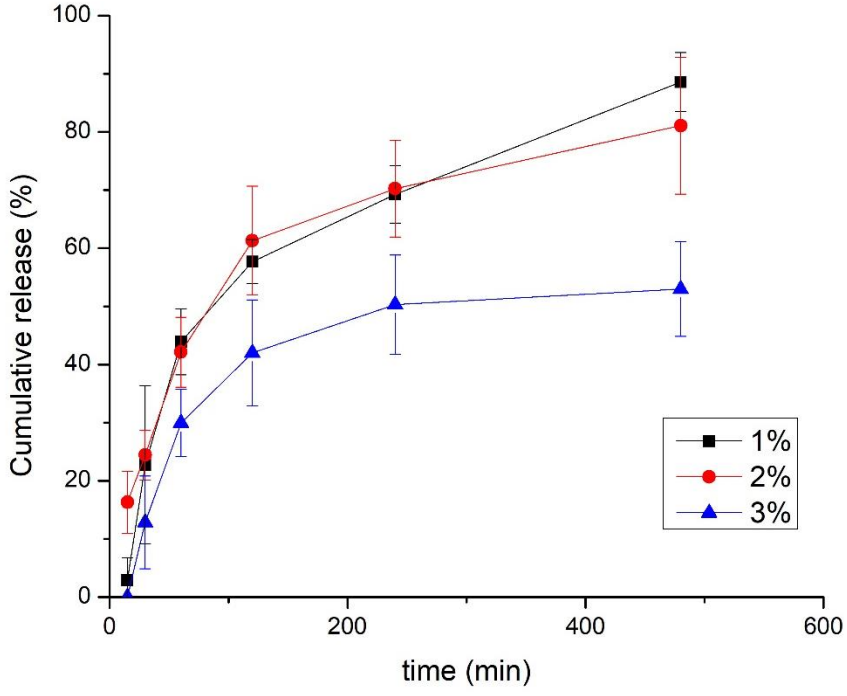
(a)



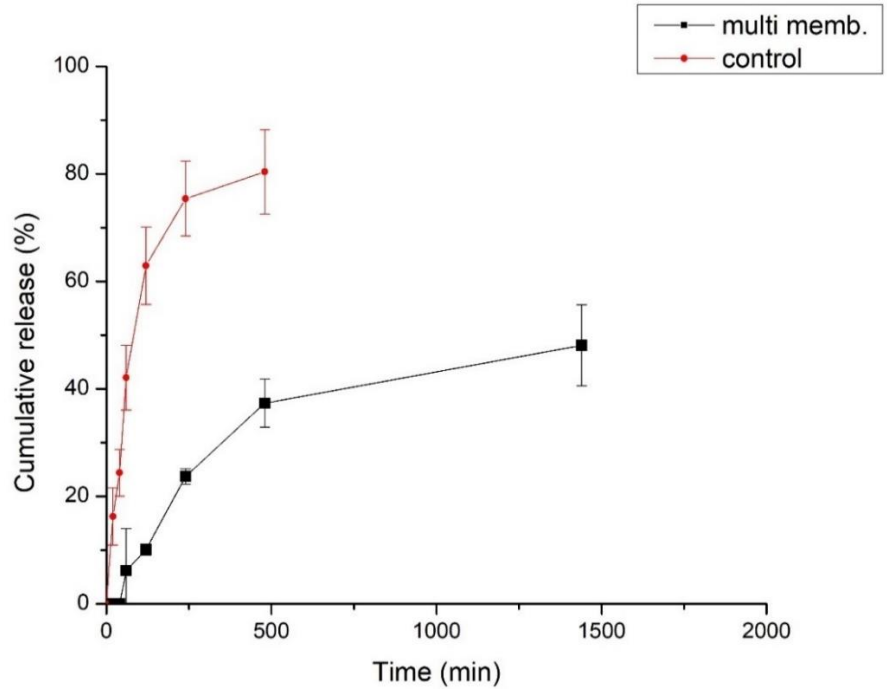
(b)


pH 1.5
pH 7.5
Cumulative release (%)
Time (mins)
0
20
40
60
80
100
0
500
1000
1500

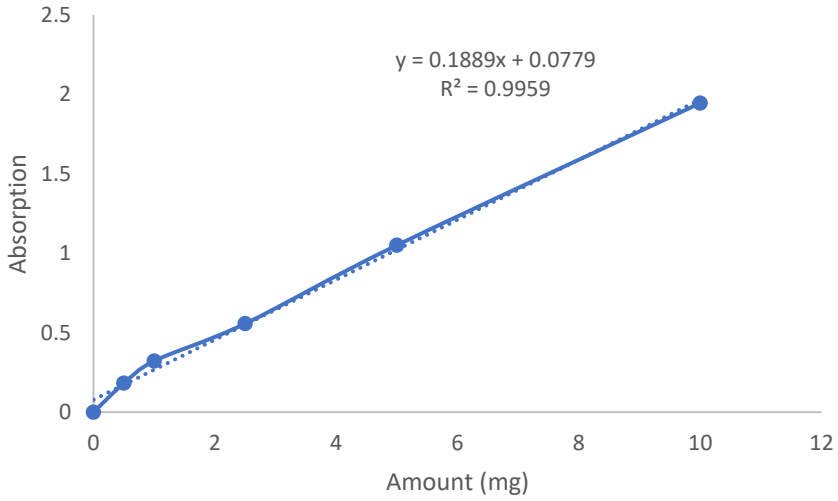
(c)


1%
2%
3%
Cumulative release (%)
time (min)
0
20
40
60
80
100
0
200
400
600

(d)



(e)



(f)

Fig. 8. a) Photograph image of chitosan hydrogels (1-6%). b) schematic illustration of working principle of pH based drug delivery in gastric-intestine system. c) Cumulative release graph of fluorescein from the hydrogel in acidic and basic pH. d) Cumulative release graph of fluorescein from the hydrogel of different concentration (1, 3 & 5%). e) Cumulative release graph of fluorescein from the hydrogel with multimembrane and no layers. f) standard curve for cumulative controlled release.

It was earlier observed that adding the multimembrane to the hydrogel prolonged their dissolution period and hence increased the life of the hydrogel in the basic conditions as per the number of layers. It was observed that more number of layers added more life to the hydrogel in the basic solution. Here, we added multimembranes to the hydrogel and then analyzed the release rates as compare to the control hydrogel. Multimembrane hydrogel laden with fluorescein was tested for release studies to see the effect of pH in prolonging the drug release time. It can be observed from fig. 8e, release rate for hydrogel system with multimembrane structure (~80%) was nearly double of the release rate for control hydrogel (~50%), with no layers. The above results show that multimembrane structures can be used where prolonged release of the drug is required. Therefore, these systems can be used for pH-sensitive and site-specific drug-delivery systems with control over different release rates depending on the number of layers.

4.6 Shape memory behavior

Shape memory materials are emerging class of materials that have capability to change their shape in response to external stimuli. Materials are said to show shape memory effect if they can be deformed into a temporary shape and can be recovered to the original shape in response to some external stimuli. The shape memory process can be sub-classified into three main steps which lead to the shape memory effect in a material. First, any material is processed or is present as its initial shape. The next step can be defined as programming the material into a desired deformed shape. This deformed temporary shape and its recovery can be achieved by application of any external stimuli such as pH, temperature, chemical solvent, stretching or compressing [247-250]. Final step is the one in which material comes back to its original shape on application of some external stimulus.

CMC solution's pH sensitivity was the main driving force for deriving potential applications in paper origami based shape memory behavior. Shape memory behavior was realized by coating CMC on one side of the filter paper thereby allowing changes in the paper in accordance with the sol-gel transition of the CMC solution. The other side of paper was made hydrophobic by applying wax to allow schematically folding and unfolding of these origamis. Different concentrations of CMC used were 3%, 5% and 10%. All these concentrations could express familiar effect on the shape memory behavior of the origamis. As shown in the Fig. 9 a and b, initial shape of the paper (i) was molded into the different shapes such as spiral coil structure and was then placed in 1 M HCl acidic solution allowing the gelation of CMC solution, thereby retaining this temporary shape (ii). Temporary shape structures were then subjected to basic solutions of 1 M NaOH solution to allow the transition from gel phase of CMC to solution phase. The gel-sol conversion helps the filter paper to come back to its initial shape (iii).

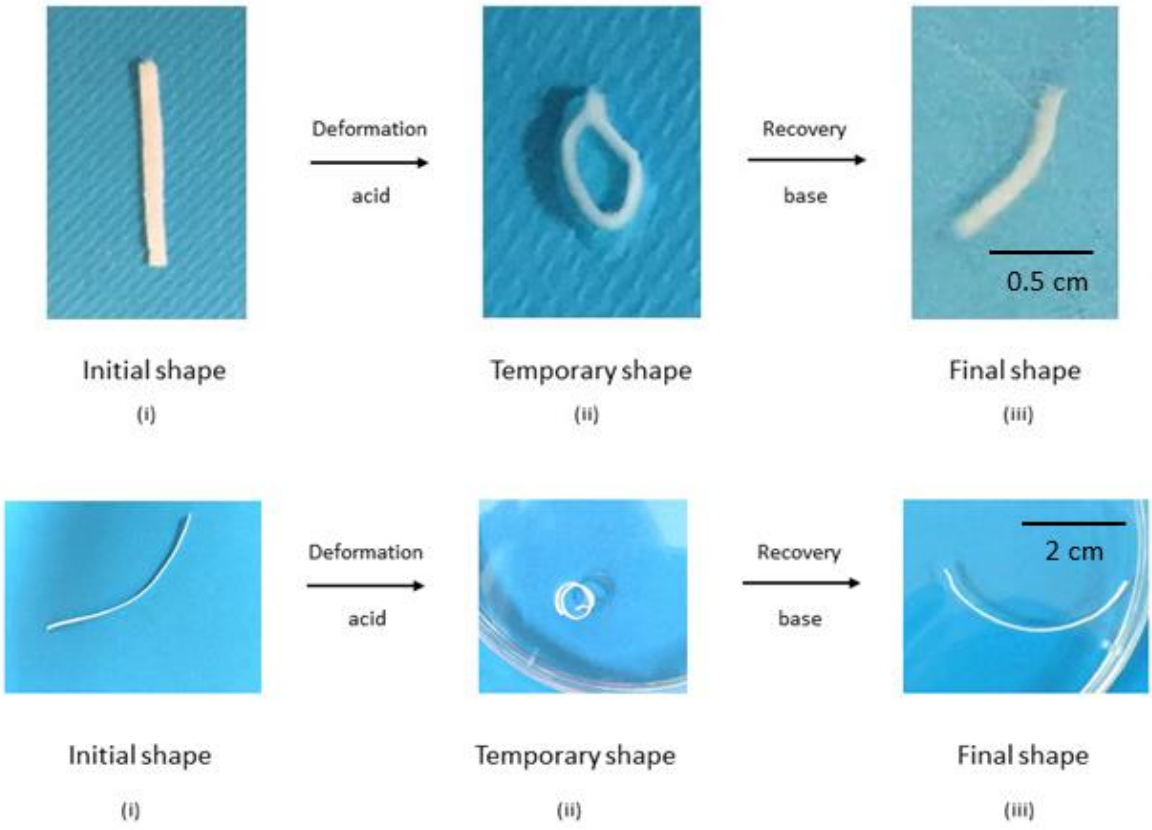


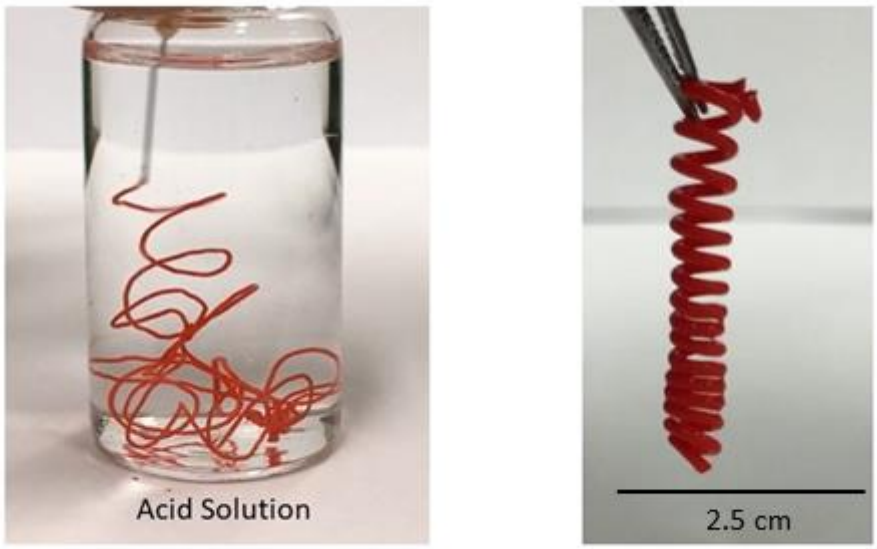
Fig. 9 Origami based shape memory behavior in response to pH solutions.

To demonstrate that it was the sol-gel conversion and not the wetting of paper which caused the shape memory behavior, the paper origamis were also tested for their change in shape in response to water solution. It was observed that there was no change in the shape of these materials and they were still in their temporary shape until they were placed in the basic solution. The above experiment was repeated to test the importance of acidic solution and was also observed as predicated to show no significant effects on the temporary shape.

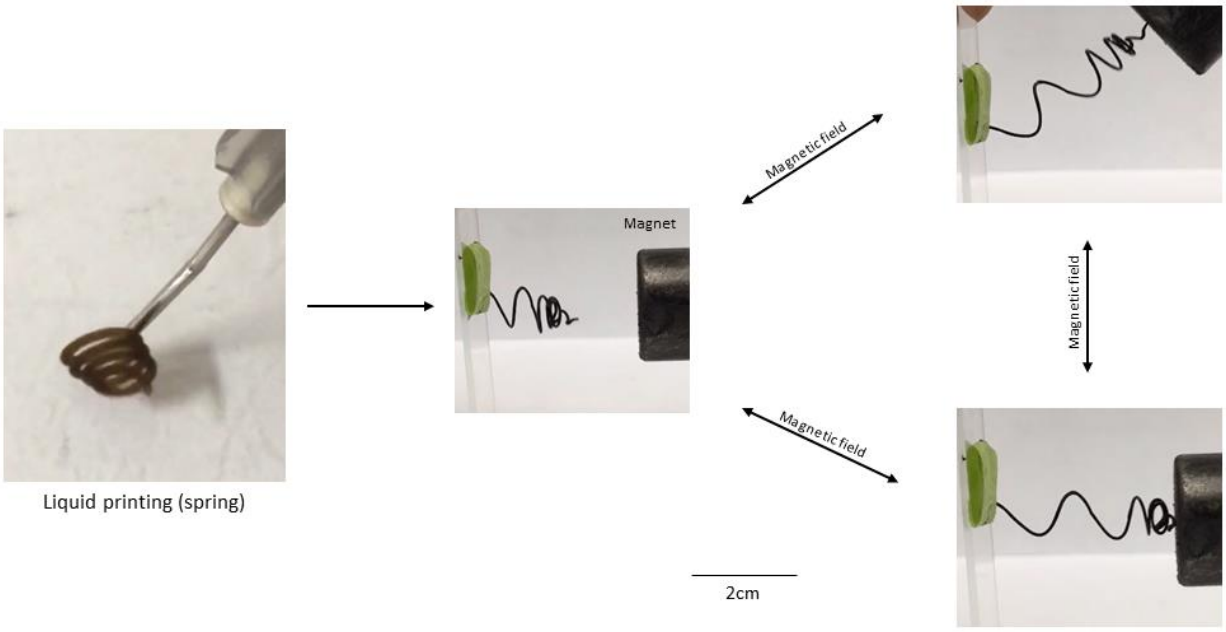
4.7 3D Printing based applications

3D printing is the modern-day technology tool which helps in upbringing and advancement of almost every research fields across the globe. pH sensitive gelation of CMC opens further door for this material to be investigated as the novel source for biomaterials based 3D printing. The capability of CMC solution to undergo phase transition in acidic solution and convert into solid phase helped in retaining the three-dimensional printed structures. CMC solution was printed on the glass substrates using conventional inkjet based 3D printing and was also found to be able to print in liquid based 3D printing (Fig. 10a).

Liquid printing is the technique in which 3D printing is used to print structures in liquid based mediums. In this case, acidic solution was used as the liquid medium for printing the CMC solutions. This technique was used to print threads and spring like structures. Magnetic nanoparticles were also mixed with the CMC solution and used for printing spring based magnetic actuators which can be moved and extended as per the applied external magnetic field (Fig. 10b).



(a)

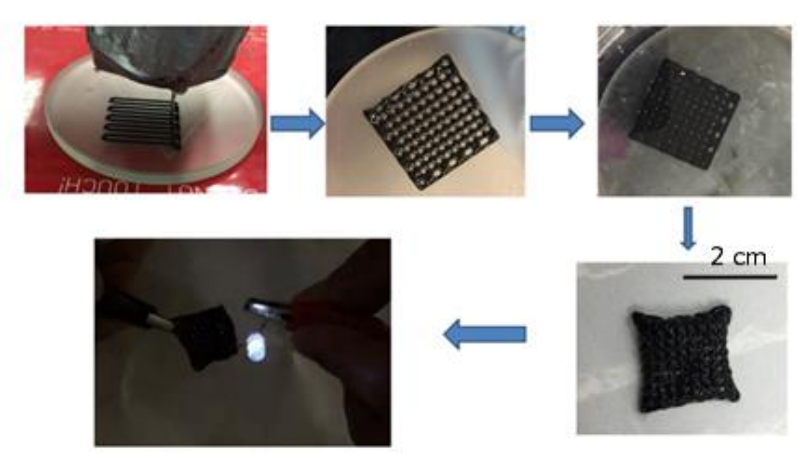


(b)

Fig. 10 Liquid printing a) CMC being liquid printed and spring like structure printed made by liquid printing; b) designing of magnetic spring based actuator and its motion controlled by external magnetic field.

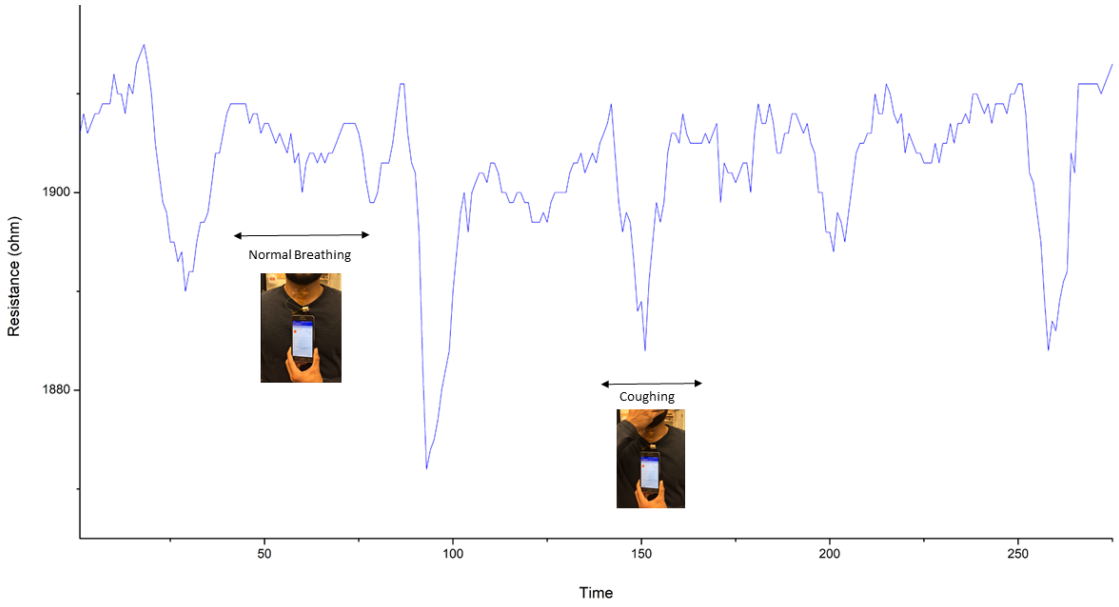
4.8 E-skin based biosensors

Another application of three-dimensional printing is flexible conductive materials. CMC solution mixed with MWCNT was used to design a conductive pattern (Fig. 11a). Conductive properties of the CMC-CNT structures were measured by testing to light a LED with one of the electrode attached to the hydrogel which was already connected to the power source and another electrode connected to the electric source completing an electric circuit which can only light the LED if the hydrogel can conduct electricity. The above circuit when completed with CMC-CNT hydrogels, could light the LED thus providing an evidence for the conductive properties of the hydrogel (Fig.11a).

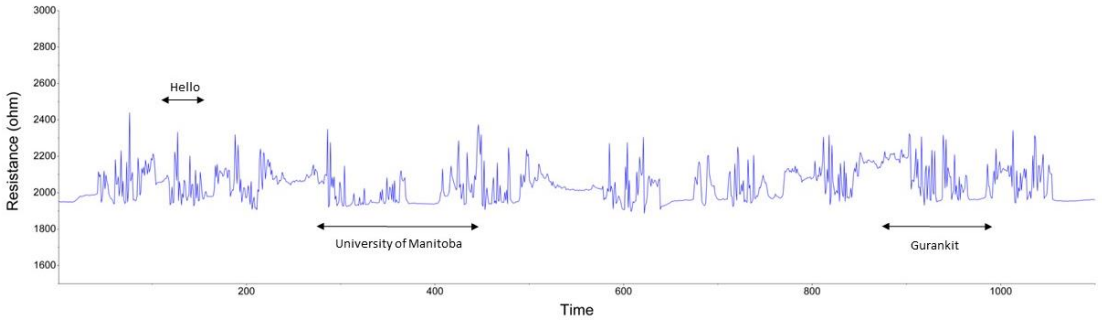


(a)

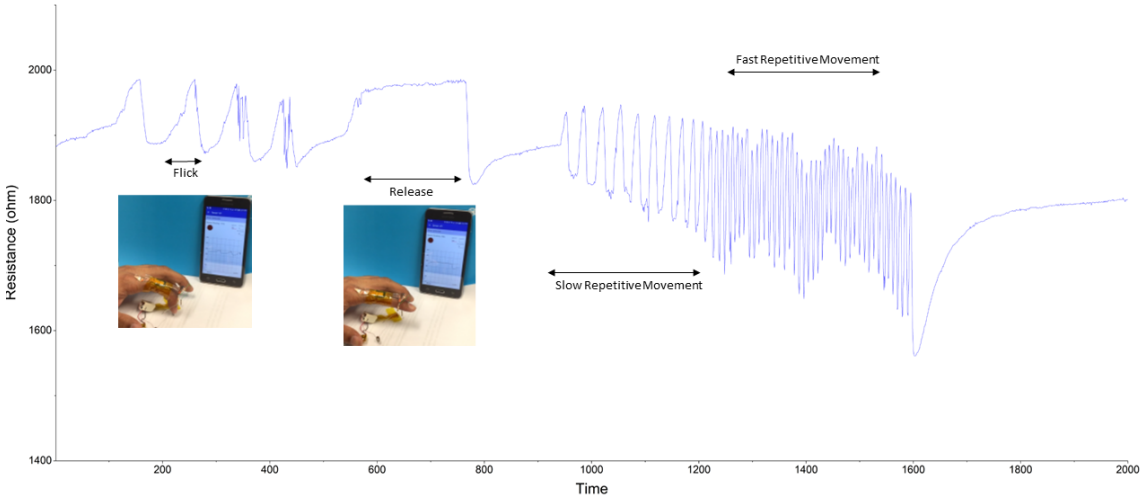
(b)


Resistance (ohm)
1900
1880
Normal Breathing
Coughing
50
100
150
200
250
Time

(c)


Resistance (ohm)
3000
2800
2600
2400
2200
2000
1800
1600
Hello
University of Manitoba
Gurankit
200
400
600
800
1000
Time

(d)


Resistance (ohm)
2000
1800
1600
1400
Flick
Release
Fast Repetitive Movement
Slow Repetitive Movement
200
400
600
800
1000
1200
1400
1600
1800
2000
Time

(e)

Fig. 11 a) process of 3D printing and crosslinking of the chitosan patterns in the shape of mesh. b) conductive mesh being bent and still shows to be conductive shows the flexibility of the material. c) real time E-skin biosensor for sensing breathing. d) E-skin biosensor as a speech sensor. E) Biosensor for sensing movements of the hand.

Flexibility was found to be another important aspect of these 3D printed conductive patterns which could widen their application areas. Conductive materials which are flexible and doesn't change their conductive properties while they are deformed or are under any external force is of great demand. The prepared conductive patterns were tested for their ability to conduct electric signals in different deformations and it was observed that, at all the orientations, the conductive mesh was still able to light the LED (Fig. 10 b). The aspect of being conductive and flexible opens the door of these materials to be used in flexible e-skin based biosensor materials. A real time wireless biosensor was then fabricated using the 3-D printed CMC-CNT conductive mesh which was connected to the wireless receiver, mobile phone (Fig. 11 c, d, e). The sensor could sense the change in applied pressure by displaying the change in resistance from the biosensor. When the sensor was used on the throat, it could sense different words and breathing by sensing the applied pressure on the sensor by these activities. The applied change in the resistance was monitored by the receiver and was displayed on the mobile screen (Fig. 11c). The sensor was used for sensing breathing as heavy breathing causes rigorous movement of throat muscles which leads to the changes in the pressure applied on the sensor which causes changes in the resistance which are then measured by the sensor. The same principle was used to sense the motion of throat muscles for sensing the words in the speech as pronouncing different words causes different applied pressure by the throat muscles on the sensor which was then used by the sensor to monitor the change in the resistance (Fig. 11d). The biosensor was also found be effective in measuring the body movement and could sense the motion of human fingers (Fig. 11e). Sensor could distinguish between the long pauses and the quick movement of the body which were sensed in real time

without any notable delay. The sensor was designed in a way in which can be used on any part to detect the body movements based on the changes in the pressure exerted by the movement of muscles on the sensor and can be displayed in real time on the mobile screen opening doors for potential applications in real time sensing in the field of health care monitoring.

4.9 Micro channels for fluid flow fabrication

Microfluidics is the art of manipulating and designing the flow of fluids at the micro scale through the channels. Microfluidics include a fluid which can flow through different channels. Here we used the two properties of the CMC hydrogels, 3D printing and sol-gel conversion to design microchannel inside the PDMS sheets to form microfluidic channels for fluidic flow through the sheets. 3D printing was used to make the patterns as per end use requirements for specific fluid flow channels. This technique provides us vast variety of channel designs as we can design any type of channels based on the inputs in the 3D printer. Sol-gel conversion was an important factor in making the channels hollow to allow the channels to be embedded in the PDMS sheet as the printed CMC hydrogel was dissolved by basic solution.

Here in we designed a Y-shaped and a 3-channel 3D printed pattern to monitor the fluid flow through the channels through the PDMS (figure 12). It can be observed from the figure 12, the liquid could flow through the channels designed in the PDMS sheet. Liquid was colored orange with the water color paint to monitor the movement of the liquid inside the channels. Flow of the liquid was dependent on the length of the channels and could be monitored by the altering the input flow rate of the liquid.

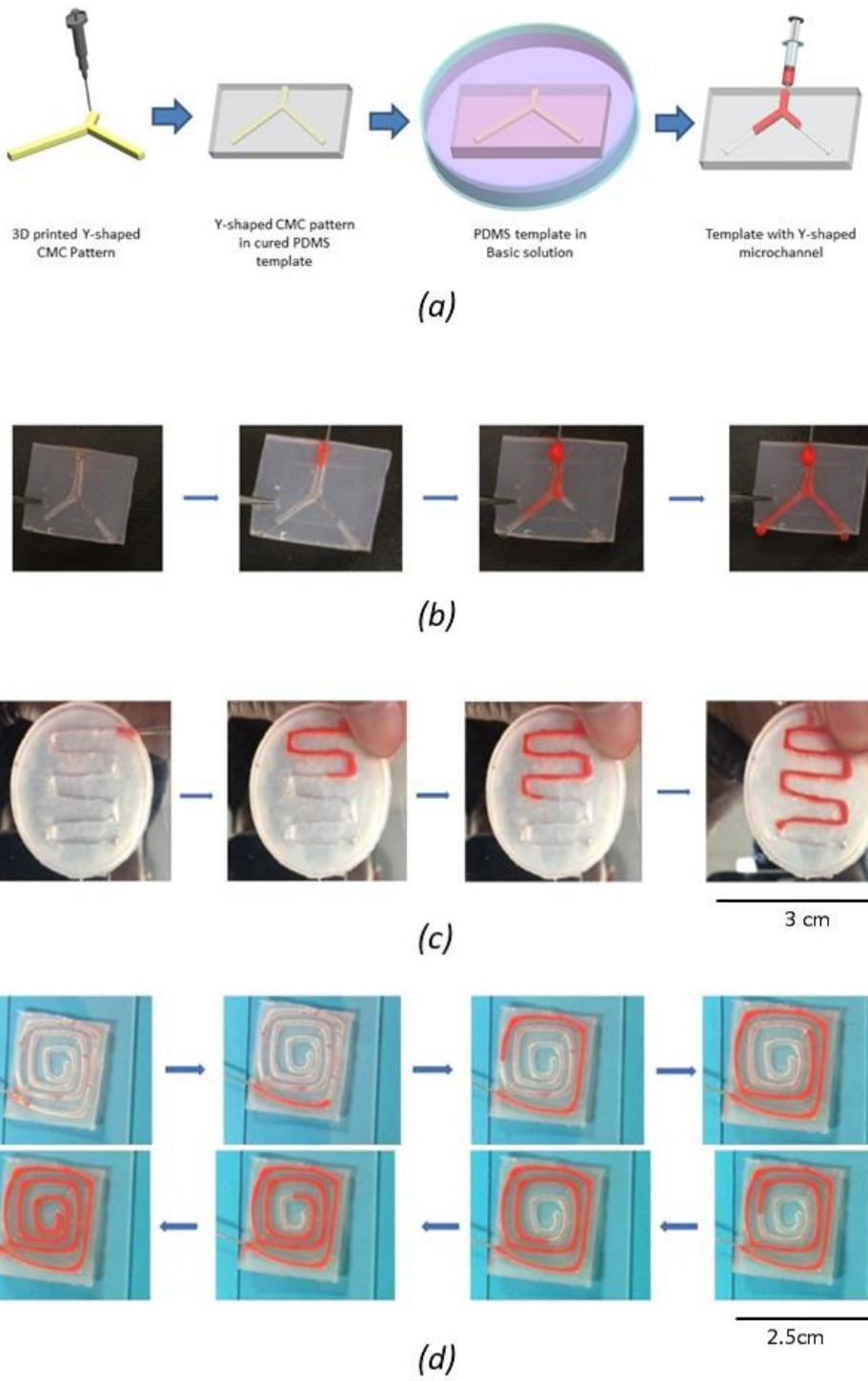


Fig. 11 Microchannels in the PDMS being tested for microfluidics showing the fluid flow inside the channels; a) Schematic illustration of the synthesis of microchannels. b) Y-shaped; c) spiral shaped; d) maze structure patterns being tested for fluid flow.

Chapter 5: Summary and Conclusions

In this report, we successfully investigated the pH-sensitive sol-gel phase conversable CMC hydrogel for intestine specific drug delivery. Furthermore, we designed the peel able onion like multimembrane hydrogels based on CMC which could mimic the onion like, and increase drastically the release time of drug in the target environment. The release rates could be further extended to various desired time intervals by modifying these hydrogel systems with multimembrane and varying the concentration of the solution for hydrogel synthesis. In addition, the versality of sol-gel property of CMC hydrogel was used in various multidisciplinary applications. Thus, pH sensitive paper origami based shape memory materials were successfully carried out. A novel bioink in 3D printing based applications and liquid 3D printing was also designed and carried out by using CMC solution. We also developed successfully e-skin based biosensors with multifunctional sensing capabilities to sense body movement, words in the speech, heavy and normal breathing by addition of MWCNT to CMC solution. Another application in the field of microfluidics was also developed by patterning microchannels into the polymers (PDMS). To our knowledge, the pH sensitive sol-gel CMC hydrogel is more suitable candidate for drug delivery, and it is also 3D printable. Thus, it is a vital biomaterial for multiple applications in medical sciences.

Chapter 6: Suggestions for Future Work

The vast field of applications which can be realized by tuning chitosan's structure is an area which needs to be explored more in detail. The applications which have been realized during this research can be further used in re shaping the present fields of sciences. The onion like structure reported in this project can be further tested for layered cell growth in the spaces between each layer. This layered cell growth can lead to the potential applications in regenerative medicine for cue-directed growth of tissues. Similarly, in-vivo studies of gene delivery and drug delivery in animal models is another potential research studies which needs to be conducted to gain more insight of the working of the CMC hydrogels systems as drug and gene delivery vehicles. Lastly, the microchannels designed by using CMC hydrogel in polymers also needs further investigation to realize applications which have not been realized. One such application which needs to be studied is the development of external temperature controlled flow of the liquids in the polymer channel systems which can be used to trigger the release of aqueous drug solutions using thermo responsive solutions as the gateways to control the flow of drug solutions inside the channels. Therefore, in a similar way there are a lot of extended potential applications which can be developed by extending the studies on the pH sensitive CMC solution.

References

1. Stuart, M.A.C., et al., *Emerging applications of stimuli-responsive polymer materials.* Nature materials, 2010. **9**(2): p. 101-113.
2. Wang, Y., et al., *Engineering nanomedicines using stimuli-responsive biomaterials.* Advanced drug delivery reviews, 2012. **64**(11): p. 1021-1030.
3. de las Heras Alarcón, C., S. Pennadam, and C. Alexander, *Stimuli responsive polymers for biomedical applications.* Chemical Society Reviews, 2005. **34**(3): p. 276-285.
4. Wei, M., et al., *Stimuli-responsive polymers and their applications.* Polymer Chemistry, 2017. **8**(1): p. 127-143.
5. Peppas, N.A., K.M. Wood, and J.O. Blanchette, *Hydrogels for oral delivery of therapeutic proteins.* Expert Opinion on Biological Therapy, 2004. **4**(6): p. 881-887.
6. Bronich, T.K., S.V. Vinogradov, and A.V. Kabanov, *Interaction of nanosized copolymer networks with oppositely charged amphiphilic molecules.* Nano Letters, 2001. **1**(10): p. 535-540.
7. Liu, R., M. Fraylich, and B.R. Saunders, *Thermoresponsive copolymers: from fundamental studies to applications.* Colloid and Polymer Science, 2009. **287**(6): p. 627-643.
8. Wang, W., et al., *Biodegradable Thermoresponsive Microparticle Dispersions for Injectable Cell Delivery Prepared Using a Single-Step Process.* Advanced Materials, 2009. **21**(18): p. 1809-1813.
9. Wu, Q., et al., *Organization of glucose-responsive systems and their properties.* Chemical reviews, 2011. **111**(12): p. 7855-7875.
10. Guo, Q., et al., *Hierarchically mesostructured porous TiO 2 hollow nanofibers for high performance glucose biosensing.* Biosensors and Bioelectronics, 2017. **92**: p. 654-660.
11. Barrett, C.J., et al., *Photo-mechanical effects in azobenzene-containing soft materials.* Soft Matter, 2007. **3**(10): p. 1249-1261.
12. Liu, Y., et al., *Humidity-and Photo-Induced Mechanical Actuation of Cross-Linked Liquid Crystal Polymers.* Advanced Materials, 2017. **29**(9).
13. Yang, C., et al., *Reduced Graphene Oxide-Containing Smart Hydrogels with Excellent Electro-Response and Mechanical Properties for Soft Actuators.* ACS Applied Materials & Interfaces, 2017. **9**(18): p. 15758-15767.
14. Gong, J., T. Nitta, and Y. Osada, *Electrokinetic modeling of the contractile phenomena of polyelectrolyte gels. One-dimensional capillary model.* The Journal of Physical Chemistry, 1994. **98**(38): p. 9583-9587.
15. Dai, T., et al., *Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects.* Expert review of anti-infective therapy, 2011. **9**(7): p. 857-879.
16. Jayakumar, R., et al., *Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications.* Progress in Materials Science, 2010. **55**(7): p. 675-709.
17. Rinaudo, M., *Chitin and chitosan: properties and applications.* Progress in polymer science, 2006. **31**(7): p. 603-632.
18. Knapczyk, J., et al., *Requirements of chitosan for pharmaceutical and biomedical applications.* Chitin and Chitosan: Sources, Chemistry, Biochemistry, Physical Properties and Applications, Elsevier, London, 1989: p. 657-663.
19. Verheul, R.J., et al., *Synthesis, characterization and in vitro biological properties of O-methyl free N, N, N-trimethylated chitosan.* Biomaterials, 2008. **29**(27): p. 3642-3649.
20. Wu, M., et al., *Preparation of N, N, N-trimethyl chitosan via a novel approach using dimethyl carbonate.* Carbohydrate Polymers, 2017. **169**: p. 83-91.
21. Kotze, A., et al., *Chitosan for enhanced intestinal permeability: prospects for derivatives soluble in neutral and basic environments.* European journal of pharmaceutical sciences, 1999. **7**(2): p. 145-

151.
22. Dong, W., et al., *Pharmacokinetics and biodegradation mechanisms of a versatile carboxymethyl derivative of chitosan in rats: in vivo and in vitro evaluation.* Biomacromolecules, 2010. **11**(6): p. 1527-1533.
23. Chen, S.-C., et al., *A novel pH-sensitive hydrogel composed of N, O-carboxymethyl chitosan and alginate cross-linked by genipin for protein drug delivery.* Journal of Controlled Release, 2004. **96**(2): p. 285-300.
24. Fei Liu, X., et al., *Antibacterial action of chitosan and carboxymethylated chitosan.* Journal of applied polymer science, 2001. **79**(7): p. 1324-1335.
25. Zhou, L., et al., *Carboxymethyl chitosan-Fe3O4 nanoparticles: preparation and adsorption behavior toward Zn2+ ions.* Acta Physico-Chimica Sinica, 2006. **22**(11): p. 1342-1346.
26. Liu, H., et al., *Thermosensitive injectable in-situ forming carboxymethyl chitin hydrogel for three-dimensional cell culture.* Acta biomaterialia, 2016. **35**: p. 228-237.
27. Agnihotri, S.A., N.N. Mallikarjuna, and T.M. Aminabhavi, *Recent advances on chitosan-based micro-and nanoparticles in drug delivery.* Journal of controlled release, 2004. **100**(1): p. 5-28.
28. Kumari, A., S.K. Yadav, and S.C. Yadav, *Biodegradable polymeric nanoparticles based drug delivery systems.* Colloids and Surfaces B: Biointerfaces, 2010. **75**(1): p. 1-18.
29. Rinaudo, M., et al., *Substituent distribution on O, N-carboxymethylchitosans by 1H and 13C NMR.* International journal of biological macromolecules, 1992. **14**(3): p. 122-128.
30. Muzzarelli, R.A., *Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone.* Carbohydrate polymers, 2009. **76**(2): p. 167-182.
31. Kim, I.-Y., et al., *Chitosan and its derivatives for tissue engineering applications.* Biotechnology advances, 2008. **26**(1): p. 1-21.
32. Ladet, S., L. David, and A. Domard, *Multi-membrane hydrogels.* Nature, 2008. **452**(7183): p. 76-79.
33. Ladet, S., et al., *Multi-membrane chitosan hydrogels as chondrocytic cell bioreactors.* Biomaterials, 2011. **32**(23): p. 5354-5364.
34. Konieczynska, M.D. and M.W. Grinstaff, *On-Demand Dissolution of Chemically Cross-Linked Hydrogels.* Accounts of Chemical Research, 2017. **50**(2): p. 151-160.
35. Ehrbar, M., et al., *Biomolecular hydrogels formed and degraded via site-specific enzymatic reactions.* Biomacromolecules, 2007. **8**(10): p. 3000-3007.
36. Li, X., et al., *Precise control and prediction of hydrogel degradation behavior.* Macromolecules, 2011. **44**(9): p. 3567-3571.
37. Ghobril, C., et al., *A dendritic thioester hydrogel based on thiol–thioester exchange as a dissolvable sealant system for wound closure.* Angewandte Chemie International Edition, 2013. **52**(52): p. 14070-14074.
38. Gyarmati, B., Á. Némethy, and A. Szilágyi, *Reversible disulphide formation in polymer networks: A versatile functional group from synthesis to applications.* European Polymer Journal, 2013. **49**(6): p. 1268-1286.
39. Baldwin, A.D. and K.L. Kiick, *Reversible maleimide–thiol adducts yield glutathione-sensitive poly (ethylene glycol)–heparin hydrogels.* Polymer chemistry, 2013. **4**(1): p. 133-143.
40. Koehler, K.C., K.S. Anseth, and C.N. Bowman, *Diels–Alder mediated controlled release from a poly (ethylene glycol) based hydrogel.* Biomacromolecules, 2013. **14**(2): p. 538-547.
41. Shanmugam, S., et al., *Natural polymers and their applications.* 2005.
42. John, M.J. and S. Thomas, *Natural Polymers: Composites*. Vol. 1. 2012: Royal Society of Chemistry.
43. Kumbar, S., C. Laurencin, and M. Deng, *Natural and synthetic biomedical polymers*. 2014: Newnes.
44. Bledzki, A. and J. Gassan, *Composites reinforced with cellulose based fibres.* Progress in polymer science, 1999. **24**(2): p. 221-274.

45. Chang, C. and L. Zhang, *Cellulose-based hydrogels: present status and application prospects.* Carbohydrate Polymers, 2011. **84**(1): p. 40-53.
46. Yu, L., K. Dean, and L. Li, *Polymer blends and composites from renewable resources.* Progress in polymer science, 2006. **31**(6): p. 576-602.
47. Ratner, B.D. and S.J. Bryant, *Biomaterials: where we have been and where we are going.* Annu. Rev. Biomed. Eng., 2004. **6**: p. 41-75.
48. Vroman, I. and L. Tighzert, *Biodegradable polymers.* Materials, 2009. **2**(2): p. 307-344.
49. Alves, N. and J. Mano, *Chitosan derivatives obtained by chemical modifications for biomedical and environmental applications.* International journal of biological macromolecules, 2008. **43**(5): p. 401-414.
50. Gupta, K.C. and M.N. RAVI KUMAR, *An overview on chitin and chitosan applications with an emphasis on controlled drug release formulations.* Journal of Macromolecular Science, Part C: Polymer Reviews, 2000. **40**(4): p. 273-308.
51. Hoffman, A.S., *Surface modification of polymers.* ♧ ✶ ⩓, 1995. **13**(3): p. 195-203.
52. Dash, M., et al., *Chitosan—A versatile semi-synthetic polymer in biomedical applications.* Progress in polymer science, 2011. **36**(8): p. 981-1014.
53. Pillai, C., W. Paul, and C.P. Sharma, *Chitin and chitosan polymers: Chemistry, solubility and fiber formation.* Progress in polymer science, 2009. **34**(7): p. 641-678.
54. Morimoto, M., H. Saimoto, and Y. Shigemasa, *Control of functions of chitin and chitosan by chemical modification.* Trends in Glycoscience and Glycotechnology, 2002. **14**(78): p. 205-222.
55. Mourya, V. and N.N. Inamdar, *Chitosan-modifications and applications: opportunities galore.* Reactive and Functional polymers, 2008. **68**(6): p. 1013-1051.
56. Bajaj, M., J. Winter, and C. Gallert, *Effect of deproteination and deacetylation conditions on viscosity of chitin and chitosan extracted from Crangon crangon shrimp waste.* Biochemical engineering journal, 2011. **56**(1-2): p. 51-62.
57. Khor, E. and L.Y. Lim, *Implantable applications of chitin and chitosan.* Biomaterials, 2003. **24**(13): p. 2339-2349.
58. Madihally, S.V. and H.W. Matthew, *Porous chitosan scaffolds for tissue engineering.* Biomaterials, 1999. **20**(12): p. 1133-1142.
59. Mao, H.-Q., et al., *Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency.* Journal of controlled release, 2001. **70**(3): p. 399-421.
60. Roy, K., et al., *Oral gene delivery with chitosan–DNA nanoparticles generates immunologic protection in a murine model of peanut allergy.* Nature medicine, 1999. **5**(4): p. 387.
61. Kyzas, G.Z., M. Kostoglou, and N.K. Lazaridis, *Relating interactions of dye molecules with chitosan to adsorption kinetic data.* Langmuir, 2010. **26**(12): p. 9617-9626.
62. Tokura, S., et al., *Induction of methamphetamine-specific antibody using biodegradable carboxymethyl-chitin.* Analytical biochemistry, 1987. **161**(1): p. 117-122.
63. Ding, F., et al., *Electrochemically induced reversible formation of carboxymethyl chitin hydrogel and tunable protein release.* New Journal of Chemistry, 2015. **39**(2): p. 1253-1259.
64. Sun, L., et al., *Conversion of crystal structure of the chitin to facilitate preparation of a 6-carboxychitin with moisture absorption–retention abilities.* Carbohydrate polymers, 2006. **66**(2): p. 168-175.
65. Yin, X., et al., *Preparation and antibacterial activity of Schiff bases from O-carboxymethyl chitosan and para-substituted benzaldehydes.* Polymer bulletin, 2012. **68**(5): p. 1215-1226.
66. Sahu, S.K., et al., *Hydrophobically modified carboxymethyl chitosan nanoparticles targeted delivery of paclitaxel.* Journal of drug targeting, 2011. **19**(2): p. 104-113.
67. Chen, X.-G. and H.-J. Park, *Chemical characteristics of O-carboxymethyl chitosans related to the preparation conditions.* Carbohydrate Polymers, 2003. **53**(4): p. 355-359.

68. Lin, Y.-H., et al., *Physically crosslinked alginate/N, O-carboxymethyl chitosan hydrogels with calcium for oral delivery of protein drugs.* Biomaterials, 2005. **26**(14): p. 2105-2113.
69. Yang, J., et al., *pH-sensitive interpenetrating network hydrogels based on chitosan derivatives and alginate for oral drug delivery.* Carbohydrate polymers, 2013. **92**(1): p. 719-725.
70. Jayakumar, R., R.L. Reis, and J.F. Mano. *Synthesis of N-carboxymethyl chitosan beads for controlled drug delivery applications*. in *Materials science forum*. 2006. Trans Tech Publ.
71. Muzzarelli, R., et al., *Reconstruction of parodontal tissue with chitosan.* Biomaterials, 1989. **10**(9): p. 598-603.
72. Guo, Z., et al., *Comparative study of the influence of active groups of chitosan derivatives on antifungal activity.* Journal of applied polymer science, 2013. **127**(4): p. 2553-2556.
73. De Campos, A.M., et al., *Chitosan nanoparticles as new ocular drug delivery systems: in vitro stability, in vivo fate, and cellular toxicity.* Pharmaceutical research, 2004. **21**(5): p. 803-810.
74. Tungtong, S., et al., *Solubility, viscosity and rheological properties of water-soluble chitosan derivatives.* Maejo International Journal of Science and Technology, 2012. **6**(2): p. 315.
75. Anitha, A., et al., *Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, O-carboxymethyl and N, O-carboxymethyl chitosan nanoparticles.* Carbohydrate Polymers, 2009. **78**(4): p. 672-677.
76. Chen, R.-N., et al., *Development of N, O-(carboxymethyl) chitosan/collagen matrixes as a wound dressing.* Biomacromolecules, 2006. **7**(4): p. 1058-1064.
77. Li, X., et al., *In situ injectable nano-composite hydrogel composed of curcumin, N, O-carboxymethyl chitosan and oxidized alginate for wound healing application.* International journal of pharmaceutics, 2012. **437**(1-2): p. 110-119.
78. Zhang, L., et al., *Blend membranes from carboxymethylated chitosan/alginate in aqueous solution.* Journal of Applied Polymer Science, 2000. **77**(3): p. 610-616.
79. Muzzarelli, R., et al., *Antimicrobial properties of N-carboxybutyl chitosan.* Antimicrobial agents and chemotherapy, 1990. **34**(10): p. 2019-2023.
80. Biagini, G., et al., *Wound management with N-carboxybutyl chitosan.* Biomaterials, 1991. **12**(3): p. 281-286.
81. Mi, F.-L., et al., *Fabrication and characterization of a sponge-like asymmetric chitosan membrane as a wound dressing.* Biomaterials, 2001. **22**(2): p. 165-173.
82. Rasad, M.S.B.A., et al., *In vitro evaluation of novel chitosan derivatives sheet and paste cytocompatibility on human dermal fibroblasts.* Carbohydrate Polymers, 2010. **79**(4): p. 1094-1100.
83. Felse, P.A. and T. Panda, *Studies on applications of chitin and its derivatives.* Bioprocess Engineering, 1999. **20**(6): p. 505-512.
84. Kogan, G., et al., *Antioxidant and antimutagenic activity of N-(2-carboxyethyl) chitosan.* Toxicology and applied pharmacology, 2004. **201**(3): p. 303-310.
85. Veleshko, A., et al., *Interaction of radionuclides with some functional derivatives of chitosan in solutions.* Radiochemistry, 2009. **51**(5): p. 490.
86. Cai, Z.-s., et al., *Synthesis, characterization and antibacterial activity of quaternized N, O-(2-carboxyethyl) chitosan.* Polymer bulletin, 2009. **62**(4): p. 445-456.
87. McKinnon, D.D., et al., *Biophysically defined and cytocompatible covalently adaptable networks as viscoelastic 3D cell culture systems.* Advanced Materials, 2014. **26**(6): p. 865-872.
88. Song, Y., H. Onishi, and T. Nagai, *Conjugate of mitomycin C with N-succinyl-chitosan: in vitro drug release properties, toxicity and antitumor activity.* International journal of pharmaceutics, 1993. **98**(1-3): p. 121-130.
89. Golyshev, A., Y.E. Moskalenko, and Y.A. Skorik, *Comparison of the acylation of chitosan with succinic anhydride in aqueous suspension and in solution.* Russian Chemical Bulletin, 2015. **64**(5):

p. 1168-1171.
90. Zhu, Q.-l., et al., *Low-density lipoprotein-coupled N-succinyl chitosan nanoparticles co-delivering siRNA and doxorubicin for hepatocyte-targeted therapy.* Biomaterials, 2014. **35**(22): p. 5965-5976.
91. Mura, C., et al., *In vitro study of N-succinyl chitosan for targeted delivery of 5-aminosalicylic acid to colon.* Carbohydrate polymers, 2011. **85**(3): p. 578-583.
92. Zhang, C., et al., *Synthesis and characterization of water-soluble O-succinyl-chitosan.* European Polymer Journal, 2003. **39**(8): p. 1629-1634.
93. Chakraborty, P., et al., *Improved mechanical and photophysical properties of chitosan incorporated folic acid gel possessing the characteristics of dye and metal ion absorption.* Journal of Materials Chemistry, 2012. **22**(38): p. 20291-20298.
94. Bao, H., et al., *Chitosan-functionalized graphene oxide as a nanocarrier for drug and gene delivery.* Small, 2011. **7**(11): p. 1569-1578.
95. Ong, S.-Y., et al., *Development of a chitosan-based wound dressing with improved hemostatic and antimicrobial properties.* Biomaterials, 2008. **29**(32): p. 4323-4332.
96. Qi, L., et al., *Preparation and antibacterial activity of chitosan nanoparticles.* Carbohydrate research, 2004. **339**(16): p. 2693-2700.
97. Sionkowska, A., et al., *Preparation and characterization of collagen/chitosan/hyaluronic acid thin films for application in hair care cosmetics.* Pure and Applied Chemistry, 2017. **89**(12): p. 1829-1839.
98. Agulló, E., et al., *Present and future role of chitin and chitosan in food.* Macromolecular Bioscience, 2003. **3**(10): p. 521-530.
99. Tan, W., et al., *Structural and functional optical imaging of three-dimensional engineered tissue development.* Tissue Engineering, 2004. **10**(11-12): p. 1747-1756.
100. Liu, Y., et al., *Cd-Aptamer Electrochemical Biosensor Based on AuNPs/CS Modified Glass Carbon Electrode.* Journal of Biomedical Nanotechnology, 2017. **13**(10): p. 1253-1259.
101. Ahsan, S.M., et al., *Chitosan as biomaterial in drug delivery and tissue engineering.* International journal of biological macromolecules, 2017.
102. Tzaneva, D., et al., *Synthesis of Carboxymethyl Chitosan and its Rheological Behaviour in Pharmaceutical and Cosmetic Emulsions.* Journal of Applied Pharmaceutical Science Vol, 2017. **7**(10): p. 070-078.
103. Hayes, E.R., *N, O-Carboxymethyl chitosan and preparative method therefor*. 1986, Google Patents.
104. Ramawat, K.G. and J.-M. Mérillon, *Polysaccharides: bioactivity and biotechnology*. 2015: Springer Heidelberg.
105. Ma, L., et al., *Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering.* Biomaterials, 2003. **24**(26): p. 4833-4841.
106. Valmikinathan, C.M., et al., *Photocrosslinkable chitosan based hydrogels for neural tissue engineering.* Soft Matter, 2012. **8**(6): p. 1964-1976.
107. Neufurth, M., et al., *Modular small diameter vascular grafts with bioactive functionalities.* PloS one, 2015. **10**(7): p. e0133632.
108. Verlee, A., S. Mincke, and C.V. Stevens, *Recent developments in antibacterial and antifungal chitosan and its derivatives.* Carbohydrate polymers, 2017. **164**: p. 268-283.
109. Mohamed, N.A., R.R. Mohamed, and R.S. Seoudi, *Synthesis and characterization of some novel antimicrobial thiosemicarbazone O-carboxymethyl chitosan derivatives.* International journal of biological macromolecules, 2014. **63**: p. 163-169.
110. Mourya, V., N.N. Inamdar, and A. Tiwari, *Carboxymethyl chitosan and its applications.* Advanced Materials Letters, 2010. **1**(1): p. 11-33.
111. Wan, A., et al., *Antioxidant activity of high molecular weight chitosan and N, O-quaternized chitosans.* Journal of agricultural and food chemistry, 2013. **61**(28): p. 6921-6928.

112. Sun, T., et al., *Antioxidant activity of N-carboxymethyl chitosan oligosaccharides.* Bioorganic & medicinal chemistry letters, 2008. **18**(21): p. 5774-5776.
113. Upadhyaya, L., et al., *Biomedical applications of carboxymethyl chitosans.* Carbohydrate polymers, 2013. **91**(1): p. 452-466.
114. Chen, Q., et al., *Carboxymethyl-chitosan protects rabbit chondrocytes from interleukin-1β-induced apoptosis.* European journal of pharmacology, 2006. **541**(1-2): p. 1-8.
115. Zhou, J., C. Elson, and T.D. Lee, *Reduction in postoperative adhesion formation and re-formation after an abdominal operation with the use of N, O-carboxymethyl chitosan.* Surgery, 2004. **135**(3): p. 307-312.
116. Zhu, A., et al., *The aggregation behavior of O-carboxymethylchitosan in dilute aqueous solution.* Colloids and Surfaces B: Biointerfaces, 2005. **43**(3-4): p. 143-149.
117. Pang, H.T., et al., *Preparation and rheological properties of deoxycholate-chitosan and carboxymethyl-chitosan in aqueous systems.* Carbohydrate Polymers, 2007. **69**(3): p. 419-425.
118. Wang, L. and A. Wang, *Adsorption behaviors of Congo red on the N, O-carboxymethyl-chitosan/montmorillonite nanocomposite.* Chemical Engineering Journal, 2008. **143**(1-3): p. 43-50.
119. Sun, S. and A. Wang, *Adsorption kinetics of Cu (II) ions using N, O-carboxymethyl-chitosan.* Journal of hazardous materials, 2006. **131**(1-3): p. 103-111.
120. Wang, M., et al., *γ-ray radiation-induced synthesis and Fe (III) ion adsorption of carboxymethylated chitosan hydrogels.* Carbohydrate polymers, 2008. **74**(3): p. 498-503.
121. Malda, J., et al., *25th anniversary article: engineering hydrogels for biofabrication.* Advanced materials, 2013. **25**(36): p. 5011-5028.
122. Tomatsu, I., K. Peng, and A. Kros, *Photoresponsive hydrogels for biomedical applications.* Advanced drug delivery reviews, 2011. **63**(14): p. 1257-1266.
123. Hong, S., et al., *3D printing of highly stretchable and tough hydrogels into complex, cellularized structures.* Advanced materials, 2015. **27**(27): p. 4035-4040.
124. Annabi, N., et al., *25th anniversary article: Rational design and applications of hydrogels in regenerative medicine.* Advanced materials, 2014. **26**(1): p. 85-124.
125. DeLouise, L.A., et al., *Hydrogel-Supported Optical-Microcavity Sensors.* Advanced Materials, 2005. **17**(18): p. 2199-2203.
126. Lee, K.Y. and D.J. Mooney, *Hydrogels for tissue engineering.* Chemical reviews, 2001. **101**(7): p. 1869-1880.
127. Ahmed, E.M., *Hydrogel: Preparation, characterization, and applications: A review.* Journal of advanced research, 2015. **6**(2): p. 105-121.
128. Peppas, N.A., et al., *Hydrogels in biology and medicine: from molecular principles to bionanotechnology.* Advanced materials, 2006. **18**(11): p. 1345-1360.
129. Peppas, N.A., et al., *Hydrogels in pharmaceutical formulations.* European Journal of Pharmaceutics and Biopharmaceutics, 2000. **50**(1): p. 27-46.
130. Ullah, F., et al., *Classification, processing and application of hydrogels: A review.* Mater Sci Eng C Mater Biol Appl, 2015. **57**: p. 414-33.
131. Kang, S.I. and Y.H. Bae, *A sulfonamide based glucose-responsive hydrogel with covalently immobilized glucose oxidase and catalase.* J Control Release, 2003. **86**(1): p. 115-21.
132. Yoshida, R., et al., *Comb-type grafted hydrogels with rapid deswelling response to temperature changes.* Nature, 1995. **374**: p. 240.
133. Chang, C., et al., *Superabsorbent hydrogels based on cellulose for smart swelling and controllable delivery.* European Polymer Journal, 2010. **46**(1): p. 92-100.
134. Sydney Gladman, A., et al., *Biomimetic 4D printing.* Nature Materials, 2016. **15**: p. 413.
135. Xia, L.-W., et al., *Nano-structured smart hydrogels with rapid response and high elasticity.* Nature Communications, 2013. **4**: p. 2226.

136. Verma, R., R.R. Adhikary, and R. Banerjee, *Smart material platforms for miniaturized devices: implications in disease models and diagnostics.* Lab on a Chip, 2016. **16**(11): p. 1978-1992.
137. Kibret, M., *Hydrogel Biomaterials.* 2011.
138. Jianqi, F. and G. Lixia, *PVA/PAA thermo-crosslinking hydrogel fiber: preparation and pH-sensitive properties in electrolyte solution.* European Polymer Journal, 2002. **38**(8): p. 1653-1658.
139. Li, J., et al., *Self-assembled supramolecular hydrogels formed by biodegradable PEO–PHB–PEO triblock copolymers and α-cyclodextrin for controlled drug delivery.* Biomaterials, 2006. **27**(22): p. 4132-4140.
140. Gupta, P., K. Vermani, and S. Garg, *Hydrogels: from controlled release to pH-responsive drug delivery.* Drug Discovery Today, 2002. **7**(10): p. 569-579.
141. Baker, J.P., et al., *Swelling equilibria for acrylamide-based polyampholyte hydrogels.* Macromolecules, 1992. **25**(7): p. 1955-1958.
142. Kashyap, N., N. Kumar, and M.N.V.R. Kumar, *Hydrogels for Pharmaceutical and Biomedical Applications.* 2005. **22**(2): p. 107-150.
143. Sadeghi, M. and H. Hosseinzadeh, *Synthesis of Starch—Poly(Sodium Acrylate-co-Acrylamide) Superabsorbent Hydrogel with Salt and pH-Responsiveness Properties as a Drug Delivery System.* Journal of Bioactive and Compatible Polymers, 2008. **23**(4): p. 381-404.
144. Tokareva, I., et al., *Nanosensors Based on Responsive Polymer Brushes and Gold Nanoparticle Enhanced Transmission Surface Plasmon Resonance Spectroscopy.* Journal of the American Chemical Society, 2004. **126**(49): p. 15950-15951.
145. Bassik, N., et al., *Photolithographically patterned smart hydrogel based bilayer actuators.* Polymer, 2010. **51**(26): p. 6093-6098.
146. Laftah, W.A., S. Hashim, and A.N. Ibrahim, *Polymer Hydrogels: A Review.* Polymer-Plastics Technology and Engineering, 2011. **50**(14): p. 1475-1486.
147. Serra, L., J. Doménech, and N.A. Peppas, *Drug transport mechanisms and release kinetics from molecularly designed poly(acrylic acid-g-ethylene glycol) hydrogels.* Biomaterials, 2006. **27**(31): p. 5440-5451.
148. Katono, H., et al., *Thermo-responsive swelling and drug release switching of interpenetrating polymer networks composed of poly(acrylamide-co-butyl methacrylate) and poly (acrylic acid).* Journal of Controlled Release, 1991. **16**(1): p. 215-227.
149. Behl, M., J. Zotzmann, and A. Lendlein, *Shape-Memory Polymers and Shape-Changing Polymers*, in *Shape-Memory Polymers*, A. Lendlein, Editor. 2010, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 1-40.
150. Wang, Y., et al., *Synthesis and preliminary photovoltaic behavior study of a soluble polyimide containing ruthenium complexes.* Polymer Chemistry, 2010. **1**(7): p. 1048-1055.
151. Geever, L.M., et al., *Characterisation and controlled drug release from novel drug-loaded hydrogels.* European Journal of Pharmaceutics and Biopharmaceutics, 2008. **69**(3): p. 1147-1159.
152. Li, Z. and J. Guan, *Thermosensitive hydrogels for drug delivery.* Expert Opinion on Drug Delivery, 2011. **8**(8): p. 991-1007.
153. Kloxin, A.M., et al., *Photodegradable hydrogels for dynamic tuning of physical and chemical properties.* Science, 2009. **324**(5923): p. 59-63.
154. Klinger, D. and K. Landfester, *Dual Stimuli-Responsive Poly(2-hydroxyethyl methacrylate-co-methacrylic acid) Microgels Based on Photo-Cleavable Cross-Linkers: pH-Dependent Swelling and Light-Induced Degradation.* Macromolecules, 2011. **44**(24): p. 9758-9772.
155. Holtz, J.H. and S.A. Asher, *Polymerized colloidal crystal hydrogel films as intelligent chemical sensing materials.* Nature, 1997. **389**(6653): p. 829-32.
156. Yan, B., et al., *Near infrared light triggered release of biomacromolecules from hydrogels loaded with upconversion nanoparticles.* J Am Chem Soc, 2012. **134**(40): p. 16558-61.

157. M., W.E., et al., *Advances in smart materials: Stimuli-responsive hydrogel thin films.* Journal of Polymer Science Part B: Polymer Physics, 2013. **51**(14): p. 1084-1099.
158. Liu, J., et al., *Dual Stimuli-Responsive Supramolecular Hydrogel Based on Hybrid Inclusion Complex (HIC).* Macromolecules, 2010. **43**(19): p. 8086-8093.
159. Liu, J., et al., *Preparation and properties of different photoresponsive hydrogels modulated with UV and visible light irradiation.* Journal of Photochemistry and Photobiology A: Chemistry, 2010. **211**(1): p. 20-25.
160. Friedrich, T., et al., *Photoisomerizable and thermoresponsive N-isopropylacrylamide-surfmer copolymer hydrogels prepared upon electrostatic self-assembly of an azobenzene bolaamphiphile.* Macromolecular rapid communications, 2013. **34**(5): p. 393-398.
161. Heller, J., *Modulated release from drug delivery devices.* Crit Rev Ther Drug Carrier Syst, 1993. **10**(3): p. 253-305.
162. Miyata, T., T. Uragami, and K. Nakamae, *Biomolecule-sensitive hydrogels.* Advanced Drug Delivery Reviews, 2002. **54**(1): p. 79-98.
163. Podual, K., F.J. Doyle, and N.A. Peppas, *Preparation and dynamic response of cationic copolymer hydrogels containing glucose oxidase.* Polymer, 2000. **41**(11): p. 3975-3983.
164. Brahim, S., D. Narinesingh, and A. Guiseppi-Elie, *Bio-smart hydrogels: co-joined molecular recognition and signal transduction in biosensor fabrication and drug delivery.* Biosensors and Bioelectronics, 2002. **17**(11): p. 973-981.
165. Miyata, T., et al., *Controlled permeation of model drugs through a bioconjugated membrane with antigen–antibody complexes as reversible crosslinks.* Polymer Journal, 2010. **42**: p. 834.
166. Feksa, L.R., et al., *Chapter 11 - Hydrogels for biomedical applications A2 - Grumezescu, Alexandru Mihai*, in *Nanostructures for the Engineering of Cells, Tissues and Organs*. 2018, William Andrew Publishing. p. 403-438.
167. Miyata, T., N. Asami, and T. Uragami, *A reversibly antigen-responsive hydrogel.* Nature, 1999. **399**: p. 766.
168. Tellis, J.C., et al., *Relative humidity sensors based on an environment-sensitive fluorophore in hydrogel films.* Anal Chem, 2011. **83**(3): p. 928-32.
169. Anderson, K.D., et al., *Responsive plasma polymerized ultrathin nanocomposite films.* Polymer, 2012. **53**(21): p. 4686-4693.
170. Wang, H.-m. and S.-x. Qu, *Constitutive models of artificial muscles: a review.* Journal of Zhejiang University-SCIENCE A, 2016. **17**(1): p. 22-36.
171. Seliktar, D., *Designing cell-compatible hydrogels for biomedical applications.* Science, 2012. **336**(6085): p. 1124-8.
172. Park, H. and K. Park, *Hydrogels in Bioapplications*, in *Hydrogels and Biodegradable Polymers for Bioapplications*. 1996, American Chemical Society. p. 2-10.
173. Jeong, B. and A. Gutowska, *Lessons from nature: stimuli-responsive polymers and their biomedical applications.* Trends in Biotechnology, 2002. **20**(7): p. 305-311.
174. Corkhill, P.H., C.J. Hamilton, and B.J. Tighe, *Synthetic hydrogels VI. Hydrogel composites as wound dressings and implant materials.* Biomaterials, 1989. **10**(1): p. 3-10.
175. Xuefeng, Y., et al., *Highly Efficient Self-Healable and Dual Responsive Cellulose-Based Hydrogels for Controlled Release and 3D Cell Culture.* Advanced Functional Materials, 2017. **27**(40): p. 1703174.
176. Jing, X., et al., *Mussel-inspired electroactive chitosan/graphene oxide composite hydrogel with rapid self-healing and recovery behavior for tissue engineering.* Carbon, 2017. **125**: p. 557-570.
177. Chai, Q., Y. Jiao, and X. Yu, *Hydrogels for Biomedical Applications: Their Characteristics and the Mechanisms behind Them.* Gels, 2017. **3**(1): p. 6.
178. Hoare, T.R. and D.S. Kohane, *Hydrogels in drug delivery: Progress and challenges.* Polymer, 2008.

49(8): p. 1993-2007.
179. Paavola, A., et al., *Controlled Release of Lidocaine from Injectable Gels and Efficacy in Rat Sciatic Nerve Block.* Pharmaceutical Research, 1995. **12**(12): p. 1997-2002.
180. Sosnik, A. and D. Cohn, *Ethoxysilane-capped PEO-PPO-PEO triblocks: a new family of reverse thermo-responsive polymers.* Biomaterials, 2004. **25**(14): p. 2851-8.
181. Cho, K.Y., et al., *Release of ciprofloxacin from poloxamer-graft-hyaluronic acid hydrogels in vitro.* International Journal of Pharmaceutics, 2003. **260**(1): p. 83-91.
182. Kim, M.R. and T.G. Park, *Temperature-responsive and degradable hyaluronic acid/Pluronic composite hydrogels for controlled release of human growth hormone.* Journal of Controlled Release, 2002. **80**(1): p. 69-77.
183. Kripa, R.R.M., et al., *Development of a Temperature-Sensitive Composite Hydrogel for Drug Delivery Applications.* Biotechnology Progress, 2006. **22**(1): p. 118-125.
184. Traitel, T., Y. Cohen, and J. Kost, *Characterization of glucose-sensitive insulin release systems in simulated in vivo conditions.* Biomaterials, 2000. **21**(16): p. 1679-1687.
185. Guiseppi-Elie, A., *Electroconductive hydrogels: Synthesis, characterization and biomedical applications.* Biomaterials, 2010. **31**(10): p. 2701-2716.
186. Shekhawat, S.S., et al., *An autoinhibited coiled-coil design strategy for split-protein protease sensors.* J Am Chem Soc, 2009. **131**(42): p. 15284-90.
187. Wilson, A.M., G. Justin, and A. Guiseppi-Elie, *Electroconductive Hydrogels*, in *Biomedical Applications of Hydrogels Handbook*, R.M. Ottenbrite, K. Park, and T. Okano, Editors. 2010, Springer New York: New York, NY. p. 319-337.
188. Kazanskii, K.S. and S.A. Dubrovskii, *Chemistry and physics of "agricultural" hydrogels*, in *Polyelectrolytes Hydrogels Chromatographic Materials*. 1992, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 97-133.
189. Jamnongkan, T. and S. Kaewpirom, *Potassium Release Kinetics and Water Retention of Controlled-Release Fertilizers Based on Chitosan Hydrogels.* Journal of Polymers and the Environment, 2010. **18**(3): p. 413-421.
190. Agaba, H., et al., *Hydrogel amendment to sandy soil reduces irrigation frequency and improves the biomass of Agrostis stolonifera.* Agricultural Sciences, 2011. **Vol.02No.04**: p. 7.
191. Demitri, C., et al., *Potential of Cellulose-Based Superabsorbent Hydrogels as Water Reservoir in Agriculture.* International Journal of Polymer Science, 2013. **2013**: p. 6.
192. Gorrasi, G., V. Bugatti, and V. Vittoria, *Pectins filled with LDH-antimicrobial molecules: Preparation, characterization and physical properties.* Carbohydrate Polymers, 2012. **89**(1): p. 132-137.
193. Müller, C.M.O., J.B. Laurindo, and F. Yamashita, *Effect of cellulose fibers addition on the mechanical properties and water vapor barrier of starch-based films.* Food Hydrocolloids, 2009. **23**(5): p. 1328-1333.
194. Gómez-Estaca, J., et al., *Biodegradable gelatin–chitosan films incorporated with essential oils as antimicrobial agents for fish preservation.* Food Microbiology, 2010. **27**(7): p. 889-896.
195. Wang, L., M.A.E. Auty, and J.P. Kerry, *Physical assessment of composite biodegradable films manufactured using whey protein isolate, gelatin and sodium alginate.* Journal of Food Engineering, 2010. **96**(2): p. 199-207.
196. Lee, S.-M., et al., *Chitosonic® Acid as a Novel Cosmetic Ingredient: Evaluation of its Antimicrobial, Antioxidant and Hydration Activities.* Materials, 2013. **6**(4): p. 1391.
197. Hardiansyah, A., et al., *Novel pH-sensitive drug carriers of carboxymethyl-hexanoyl chitosan (Chitosonic[registered sign] Acid) modified liposomes.* RSC Advances, 2015. **5**(30): p. 23134-23143.
198. Dalaran, M., et al., *Study on a novel polyampholyte nanocomposite superabsorbent hydrogels: Synthesis, characterization and investigation of removal of indigo carmine from aqueous solution.* Desalination, 2011. **279**(1): p. 170-182.

199. Guibal, E., *Interactions of metal ions with chitosan-based sorbents: a review.* Separation and Purification Technology, 2004. **38**(1): p. 43-74.
200. Hill-West, J.L., et al., *Inhibition of thrombosis and intimal thickening by in situ photopolymerization of thin hydrogel barriers.* Proceedings of the National Academy of Sciences, 1994. **91**(13): p. 5967-5971.
201. Jeong, B., et al., *New biodegradable polymers for injectable drug delivery systems.* Journal of Controlled Release, 1999. **62**(1-2): p. 109-114.
202. Jeong, B., S.W. Kim, and Y.H. Bae, *Thermosensitive sol–gel reversible hydrogels.* Advanced drug delivery reviews, 2012. **64**: p. 154-162.
203. Khanh, N.M. and L.D. Sung, *Injectable Biodegradable Hydrogels.* Macromolecular Bioscience, 2010. **10**(6): p. 563-579.
204. Huynh, C.T., M.K. Nguyen, and D.S. Lee, *Injectable Block Copolymer Hydrogels: Achievements and Future Challenges for Biomedical Applications.* Macromolecules, 2011. **44**(17): p. 6629-6636.
205. Singh, N.K. and D.S. Lee, *In situ gelling pH-and temperature-sensitive biodegradable block copolymer hydrogels for drug delivery.* Journal of Controlled Release, 2014. **193**: p. 214-227.
206. Lu, C., et al., *Micellization and gelation of aqueous solutions of star-shaped PEG–PCL block copolymers consisting of branched 4-arm poly(ethylene glycol) and polycaprolactone blocks.* European Polymer Journal, 2007. **43**(5): p. 1857-1865.
207. Liu, L., et al., *In situ forming hydrogels based on chitosan for drug delivery and tissue regeneration.* Asian Journal of Pharmaceutical Sciences, 2016. **11**(6): p. 673-683.
208. Jeong, B., et al., *Biodegradable block copolymers as injectable drug-delivery systems.* Nature, 1997. **388**: p. 860.
209. Lee, J.Y., et al., *In vivo efficacy of paclitaxel-loaded injectable in situ-forming gel against subcutaneous tumor growth.* International Journal of Pharmaceutics, 2010. **392**(1): p. 51-56.
210. Loh, X.J. and J. Li, *Biodegradable thermosensitive copolymer hydrogels for drug delivery.* Expert Opinion on Therapeutic Patents, 2007. **17**(8): p. 965-977.
211. Vermonden, T., R. Censi, and W.E. Hennink, *Hydrogels for Protein Delivery.* Chemical Reviews, 2012. **112**(5): p. 2853-2888.
212. Liu, L., et al., *Smart gelation of chitosan solution in the presence of NaHCO3 for injectable drug delivery system.* International Journal of Pharmaceutics, 2011. **414**(1): p. 6-15.
213. Li, F., et al., *A new injectable in situ forming hydroxyapatite and thermosensitive chitosan gel promoted by Na2CO3.* Soft Matter, 2014. **10**(13): p. 2292-2303.
214. Jakus, A.E., A.L. Rutz, and R.N. Shah, *Advancing the field of 3D biomaterial printing.* Biomedical Materials, 2016. **11**(1): p. 014102.
215. Melchels, F.P.W., J. Feijen, and D.W. Grijpma, *A review on stereolithography and its applications in biomedical engineering.* Biomaterials, 2010. **31**(24): p. 6121-6130.
216. Duan, B. and M. Wang, *Selective laser sintering and its application in biomedical engineering.* MRS bulletin, 2011. **36**(12): p. 998-1005.
217. Lee, C.H., et al., *Regeneration of the articular surface of the rabbit synovial joint by cell homing: a proof of concept study.* The Lancet, 2010. **376**(9739): p. 440-448.
218. Jakus, A.E., et al., *Three-Dimensional Printing of High-Content Graphene Scaffolds for Electronic and Biomedical Applications.* ACS Nano, 2015. **9**(4): p. 4636-4648.
219. Franco, J., et al., *Direct write assembly of calcium phosphate scaffolds using a water-based hydrogel.* Acta biomaterialia, 2010. **6**(1): p. 218-228.
220. Michna, S., W. Wu, and J.A. Lewis, *Concentrated hydroxyapatite inks for direct-write assembly of 3-D periodic scaffolds.* Biomaterials, 2005. **26**(28): p. 5632-5639.
221. Placone, J.K. and A.J. Engler, *Recent Advances in Extrusion-Based 3D Printing for Biomedical Applications.* Advanced healthcare materials, 2018. **7**(8): p. 1701161.

222. Liu, W., et al., *Extrusion Bioprinting of Shear-Thinning Gelatin Methacryloyl Bioinks.* Advanced healthcare materials, 2017. **6**(12).
223. Tsai, Y.-C., et al., *Synthesis of thermoresponsive amphiphilic polyurethane gel as a new cell printing material near body temperature.* ACS applied materials & interfaces, 2015. **7**(50): p. 27613-27623.
224. Skardal, A., et al., *A hydrogel bioink toolkit for mimicking native tissue biochemical and mechanical properties in bioprinted tissue constructs.* Acta biomaterialia, 2015. **25**: p. 24-34.
225. Blaeser, A., et al., *Controlling shear stress in 3D bioprinting is a key factor to balance printing resolution and stem cell integrity.* Advanced healthcare materials, 2016. **5**(3): p. 326-333.
226. Highley, C.B., C.B. Rodell, and J.A. Burdick, *Direct 3D printing of shear-thinning hydrogels into self-healing hydrogels.* Advanced Materials, 2015. **27**(34): p. 5075-5079.
227. Nguyen, D.G., et al., *Bioprinted 3D primary liver tissues allow assessment of organ-level response to clinical drug induced toxicity in vitro.* PloS one, 2016. **11**(7): p. e0158674.
228. Kang, H.-W., et al., *A 3D bioprinting system to produce human-scale tissue constructs with structural integrity.* Nature biotechnology, 2016. **34**(3): p. 312.
229. Liu, W., et al., *Rapid continuous multimaterial extrusion bioprinting.* Advanced Materials, 2017. **29**(3).
230. Demirtaş, T.T., G. Irmak, and M. Gümüşderelioğlu, *A bioprintable form of chitosan hydrogel for bone tissue engineering.* Biofabrication, 2017. **9**(3): p. 035003.
231. Magalhães, J., et al., *Chapter 16 - Oral Administration of Nanoparticles-Based TB Drugs A2 - Grumezescu, Alexandru Mihai*, in *Multifunctional Systems for Combined Delivery, Biosensing and Diagnostics*. 2017, Elsevier. p. 307-326.
232. Patel, A., et al., *Colon targeted drug delivery system: a review system.* Journal of pharmaceutical science and bioscientific research, 2011. **1**(1): p. 37-49.
233. Li, L., et al., *Preparation of chitosan-based multifunctional nanocarriers overcoming multiple barriers for oral delivery of insulin.* Materials Science and Engineering: C, 2017. **70**: p. 278-286.
234. Hou, L., et al., *Smart nanocomposite hydrogels based on azo crosslinked graphene oxide for oral colon-specific drug delivery.* Nanotechnology, 2016. **27**(31): p. 315105.
235. Sinha, V. and R. Kumria, *Polysaccharides in colon-specific drug delivery.* International journal of pharmaceutics, 2001. **224**(1-2): p. 19-38.
236. Behr, J.P., *Synthetic gene-transfer vectors.* Accounts of Chemical Research, 1993. **26**(5): p. 274-278.
237. Mulligan, R.C., *The basic science of gene therapy.* Science, 1993. **260**(5110): p. 926-932.
238. Dull, T., et al., *A third-generation lentivirus vector with a conditional packaging system.* Journal of virology, 1998. **72**(11): p. 8463-8471.
239. Yin, H., et al., *Non-viral vectors for gene-based therapy.* Nature Reviews Genetics, 2014. **15**(8): p. 541.
240. MacLaughlin, F.C., et al., *Chitosan and depolymerized chitosan oligomers as condensing carriers for in vivo plasmid delivery.* Journal of Controlled Release, 1998. **56**(1-3): p. 259-272.
241. Song, Q., et al., *Synthesis and property studies of N-carboxymethyl chitosan.* Journal of Applied Polymer Science, 2011. **119**(6): p. 3282-3285.
242. Kalliola, S., et al., *The pH sensitive properties of carboxymethyl chitosan nanoparticles cross-linked with calcium ions.* Colloids and Surfaces B: Biointerfaces, 2017. **153**: p. 229-236.
243. Friedman, M., *Applications of the Ninhydrin Reaction for Analysis of Amino Acids, Peptides, and Proteins to Agricultural and Biomedical Sciences.* Journal of Agricultural and Food Chemistry, 2004. **52**(3): p. 385-406.
244. Lee, S.H., et al., *Enzyme-mediated cross-linking of Pluronic copolymer micelles for injectable and in situ forming hydrogels.* Acta Biomaterialia, 2011. **7**(4): p. 1468-1476.
245. Choi, B., et al., *Introduction to in situ forming hydrogels for biomedical applications*, in *In-Situ Gelling Polymers*. 2015, Springer. p. 5-35.

246. Wu, J., Z.-G. Su, and G.-H. Ma, *A thermo-and pH-sensitive hydrogel composed of quaternized chitosan/glycerophosphate.* International Journal of Pharmaceutics, 2006. **315**(1): p. 1-11.
247. Lendlein, A. and S. Kelch, *Shape-memory polymers.* Angewandte Chemie International Edition, 2002. **41**(12): p. 2034-2057.
248. Peraza-Hernandez, E.A., et al., *Origami-inspired active structures: a synthesis and review.* Smart Materials and Structures, 2014. **23**(9): p. 094001.
249. Zhang, X., et al., *Optically-and thermally-responsive programmable materials based on carbon nanotube-hydrogel polymer composites*. 2011, CALIFORNIA UNIV BERKELEY DEPT OF ELECTRICAL ENGINEERING AND COMPUTER SCIENCE.
250. Qi, X., et al., *Water-induced shape memory effect of graphene oxide reinforced polyvinyl alcohol nanocomposites.* Journal of Materials Chemistry A, 2014. **2**(7): p. 2240-2249.