Nisco Engineering AG

HYDROGELS AND NISCO TECHNOLOGIES FOR ENCAPSULATION

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Content

1. **Hydrogels a theoretic introduction**

Hydrogels are hydrophilic polymer networks which may absorb from 10ï 20% (an arbitrary lower limit) up to thousands of times their dry weight in water. Hydrogels are one of the upcoming classes of polymer-based systems that embrace numerous biomedical and pharmaceutical applications. Because of their inherent property of biocompatibility they offer good opportunities as protein delivery systems or tissue engineering scaffolds. Their hydrophilic, soft and rubbery nature ensures minimal tissue irritation and a low tendency of cells and proteins to adhere to the hydrogel surface. [1]

There is vast potential of different applications, including soil/water stabilization layers in farming and civil engineering structures, soil conditioners, controlled release of fertilizers, fiber and metallic cable sealing, in water technologies, in cosmetics, in drug delivery systems, in ótissue engineeringôas matrices for repairing and regenerating a wide variety of tissues and organs and in many other fields. [1]

The use of hydrogel for biomedical applications dates back to 1960 when Wichterle and Lim developed crosslinked poly (hydroxyethyl methacrylate) (pHEMA). First synthetic hydrogels of HEMA with EGDMA (Ethylene glycol di-methyl acrylate) as cross-linker were prepared for biological use and later used for production of contact lenses. [2]

Hydrogels may be chemically stable or they may degrade and eventually disintegrate and dissolve. They are called ó reversibleô or ó physicalôgels when the networks are held together by molecular entanglements, and/or secondary forces including ionic, H-bonding or hydrophobic forces. [3,4]

Physical hydrogels are not homogeneous, since clusters of molecular entanglements, or hydrophobically- or ionically-associated domains, can create inhomogeneities. Free chain ends or chain loops also represent transient network defects in physical gels.

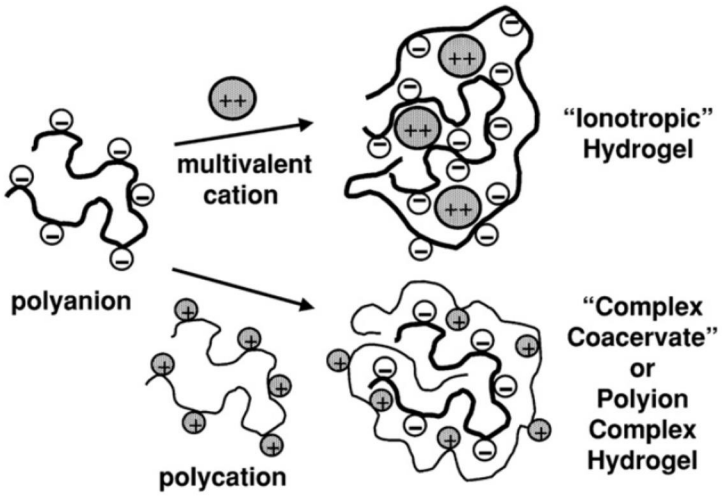

Fig. 1: Schematic of methods for formation of two types of ionic hydrogels

When a polyelectrolyte is combined with a multivalent ion of the opposite charge, it may form a physical hydrogel known as an ó ionotropicôhydrogel. Calcium alginate is an example of this type of hydrogel. Further, when polyelectrolytes of opposite charges are mixed, they may gel or precipitate depending on their concentrations, the ionic strength, and pH of the solution. The products of such ion-crosslinked systems are known as complex coacervates, polyion

complexes, or polyelectrolyte complexes. Complex coacervates and polyion complex hydrogels have become attractive as tissue engineering matrices. All of these interactions are reversible, and can be disrupted by changes in physical conditions such as ionic strength, pH, temperature, application of stress, or addition of specific solutes that compete with the polymeric ligand for the affinity site on the protein. An example of a polyionic hydrogel is a complex of alginic acid and polylysine (PLL). [5]

Hydrogels are called ópermanentô or ôchemicalô gels when they are covalently-crosslinked networks. The synthetic hydrogels of Wichterle and Lim [1] were based on copolymerization of HEMA with the crosslinker EGDMA (HEMA, hydroxyethyl methacrylate, EGDMA, ethylene glycol dimethacrylate). Chemical hydrogels may also be generated by crosslinking of water soluble polymers, or by conversion of hydrophobic polymers to hydrophilic polymers plus crosslinking to form a network. Sometimes in the latter case crosslinking is not necessary. The functional groups can stabilize the hydrogel by hydrophobic interactions, thus forming a physical hydrogel.

In the crosslinked state, crosslinked hydrogels reach an equilibrium swelling level in aqueous solutions which depends mainly on the crosslink density (estimated by the MW between crosslinks, Mc). Like physical hydrogels, chemical hydrogels are not homogeneous. They usually contain regions of low water swelling and high crosslink density, called ôclustersô that are dispersed within regions of high swelling, and low crosslink density.

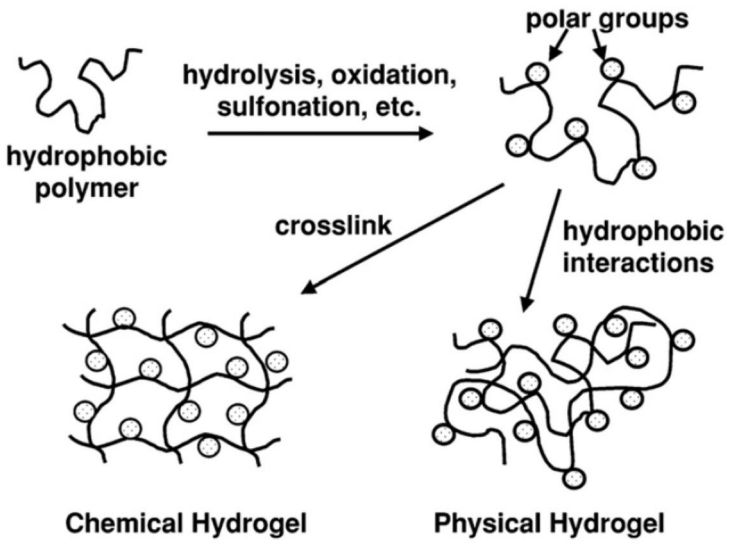

Fig. 2: Schematic of methods for formation of hydrogels by chemical modification of hydrophobic polymers [5]

Hydrogels may have many different physical forms, including:

a. solid molded forms (e.g., soft contact lenses),
b. pressed powder matrices (e.g., pills or capsules for oral ingestion),
c. microparticles (e.g., as bioadhesive carriers or wound treatments),
d. coatings (e.g., on implants or catheters; on pills or capsules; or coatings on the inside capillary wall in capillary electrophoresis),
e. membranes or sheets (e.g., as a reservoir in a transdermal drug delivery patch; or for 2D electrophoresis gels),
f. encapsulated solids (e.g., in osmotic pumps), and
g. liquids (e.g., that form gels on heating or cooling).

The different hydrogel compositions can be divided into natural polymer hydrogels, synthetic polymer hydrogels and combinations of the two classes.

Natural hydrogel polymers

Agarose

Agarose is a polysaccharide derived from the cell wall of a group of red algae (*Rhodophyceae*), including *Gelidium* and *Gracilaria* [30]. The plant is harvested and agarose is extracted after a series of purification and homogenization steps [55]. The main structure of agarose consists of alternating units of ɓ-D-galactopyranose and 3,6-anhydro-Ŭ-L-galactopyranose. Agarose extracted from different sources can have different chemical compositions; for example, sulfates can be found instead of the hydroxyl groups with a variable degree of substitution. Agarose is a responsive polymer and its aqueous solutions undergo a solï gel transition upon cooling. Above the solï gel temperature, agarose exhibits a random-coil conformation in solution, and upon cooling the structure changes to a double helix. Some of the helices then aggregate and the hydrogen bonds between structural water and galactose stabilize the structure. The gelling temperature depends on the concentration of the solution, the average molecular weight of the polymer and its structure. For this reason, there is a wide range of commercially available agarose, characterized by different gel strengths and solï gel transition temperatures. Some of them can be used for cell encapsulation since their solï gel transition occurs at around 37°C. The thermal solï gel transition of agarose is reversible and presents a marked thermal hysteresis, which is a wide temperature difference between gelling and liquefaction. [24].

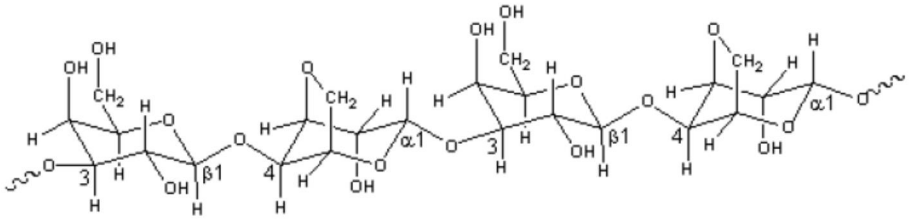

Fig. 3: Structures of agarose [30]

The average pore size of agarose hydrogels and, as a consequence, the mass transport properties are influenced by the concentration of the polymer in solution and the settling temperature. An increase in concentration results in tightly packed helices that translate to a decrease in pore size. [31]

Agarose does not provide adhesion motifs to cells and does not allow interaction between adherent cells and the entrapping matrix. However, it can be supplemented with adhesion molecules of the extracellular matrix, such as fibronectin or RGD (Arg-Gly-Asp)soluble peptide. Agarose is not biodegradableð it can only be degraded by specific bacteria, not mammals. It can be degraded *in vitro* by agarases. [24]

Agarose is used widely in molecular biology and since it is well accepted following implantation it has been evaluated for immunoisolation purposes. The non-degradable nature of the gel, however, means that it has not been widely used in tissue engineering since scaffold materials used in tissue engineering applications should degrade over time to allow space for accumulation of new tissue. [14]

Agarose forms thermoreversible gels when dissolved in water and plays the major role in the mechanical behavior of aqueous agar-agar gels. The gelation can take place only when parts

of the chains form ordered regions (junctions). In organic solvents, agarose is normally insoluble and cannot form gels. Apparently, the gelling possibility in aqueous solutions is also a consequence of the structure of water.

A characteristic feature of agarose is that the gels show massive thermal hysteresis, attributed to the formation of large aggregates that remain stable at temperatures much higher than those at which individual helices form on cooling. [37]

Alginate

Alginate is a natural polysaccharide isolated from brown algae and bacteria which has been used with great success as a wound dressing and as a food additive. On dissolution in an aqueous medium, alginate forms a hydrocolloid, which gels ionotropically following the addition of multivalent cations. [14]

Alginate contains blocks of (1ï 4)-linked **ß**-D-mannuronic acid (M) and **Ŭ**-L-guluronic acid (G) monomers (Fig. 4a). Typically, the blocks are composed of three different forms of polymer segments: consecutive G residues, consecutive M residues and alternating MG residues.

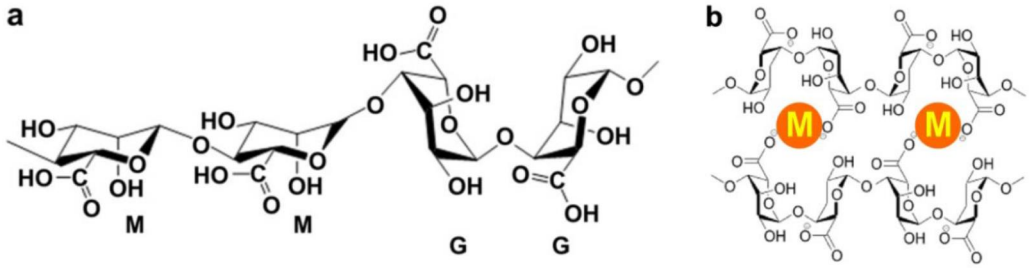

Fig. 4: (a) Chemical structure of alginate; (b) Mechanism of ionic interaction between alginate and divalent cations. [15]

An alginate gel will develop instantaneously in the presence of divalent cations like Ca^{2+}, Ba^{2+} or Sr^{2+} and acid gels may also develop at low pH. Gelling occurs when the divalent cations take part in the interchain ionic binding between guluronic acids blocks (G-blocks) in the polymer chain giving rise to a three dimensional network. Such binding zones between the G-blocks are often referred to as egg-boxes, and consequently alginates with a high content of G-blocks induce stronger gels. Gels made of M-rich alginate are softer and more fragile, and may also have a lower porosity. This is due to the lower binding strength between the polymer chains and to the higher flexibilities of the molecules.

Alginate is of particular interest for a broad range of applications as a biomaterial and especially as the supporting matrix or delivery system for tissue repair and regeneration. Due to its outstanding properties in terms of biocompatibility, biodegradability, non-antigenicity and chelating ability, alginate has been widely used in a variety of biomedical applications including tissue engineering, drug delivery and in some formulations preventing gastric reflux. As a result of the naturally occurring polysaccharide, alginate exhibits a pH-dependent anionic nature and has the ability to interact with cationic polyelectrolytes and proteoglycans. Therefore, delivery systems for cationic drugs and molecules can be obtained through simple electrostatic interactions.

Scaffolds are often used for the delivery of drugs, growth factors and therapeutically useful cells. As such, scaffolding materials allow protection of biologically active substances or cells from the biological environment.

As a U.S. Food and Drug Administration (FDA)-approved polymer, alginate has become one of the most important biomaterials for diverse applications in regeneration medicine, nutrition supplements, semipermeable separation and other areas. [15]

The degradation of alginate is slow and uncontrollable, which is undesirable for tissue engineering applications. To overcome this problem, previous studies developed alginate hydrogels that were hydrolytically degradable via partial oxidation of the alginate. [18]

Sodium alginate reaction with $CaCl_2$

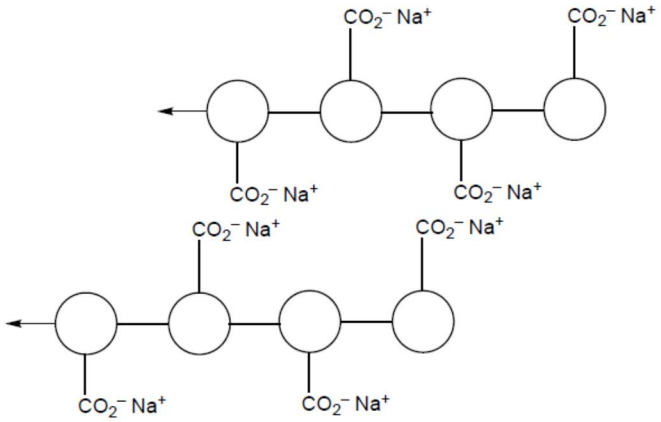

Fig. 5: Schematic of sodium alginate polymer

When sodium alginate is put into a solution of calcium ions, the calcium ions replace the sodium ions in the polymer. Each calcium ion can attach to two of the polymer strands and the cross-linking occurs:

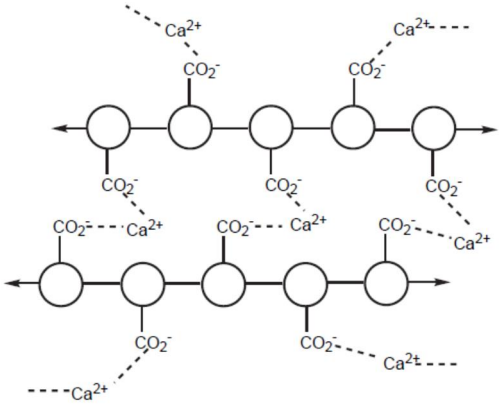

Fig. 6: Schematic of alginate polymer in $CaCl_2$ solution [16]

In contrast to most other polysaccharide gels, alginate gels can develop and set at constant temperature. This unique property is particularly useful in applications involving fragile materials like cells or tissue with low tolerance for higher temperatures.

Hyaluronic acid (HA)

Hyaluronic acid is a polysaccharide present in all living organisms and is found in most connective tissues. It is a glycosaminoglycan synthesized by membrane-bound hyaluronan synthases, which distinguishes it from other glycosaminoglycans that are produced in the Golgi apparatus. Hyaluronic acid can be obtained from many tissues by extraction or enzymatic digestion and it can also be produced by bacteria. Different sources and extraction protocols can lead to preparations of hyaluronic acid characterized by similar molecular weight but different content of endotoxins and contaminating proteins, leading to a different behavior in *in vivo* and *in vitro* experiments.

Hyaluronic acid is a linear anionic polysaccharide comprising 1,3-ß-D-glucuronic acid and 1,4-ß-N-acetyl-D-glucosamine, a structure conserved in all mammals. It is a hydrophilic polymer that can form highly viscous solutions at low concentrations it is widely used as a lubricant and in preventing postsurgical adhesions. The molecule of hyaluronic acid in solution is stiffened by a combination of the chemical structure of the disaccharide, internal hydrogen bonds and interactions with the solvent. [25]

The polymer has to be chemically modified to form a hydrogel. This procedure may involve the modification of the carboxyl or the hydroxyl group by esterification and cross-linking with, for example, glutaraldehyde, carbodiimide and divinyl sulfone.

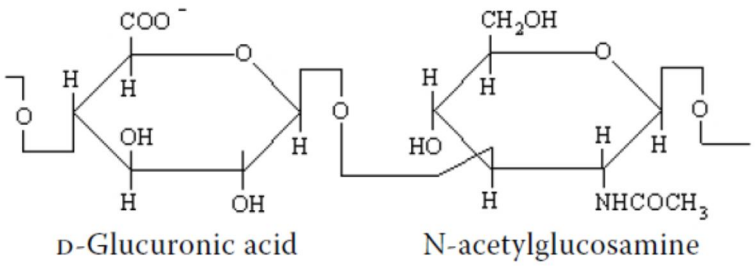

Fig. 7**:** Chemical structure of HA [25]

Hyaluronic acid is unique because of its viscoelastic and hydrodynamic properties, its assembly into extracellular and pericellular matrices, and its effects on cell signaling. Its use as a biomaterial has been driven largely by its physical properties and viscoelastic behavior.

The biological properties of HA and its fragments have largely been ignored in its use as a biomaterial. As HA is more widely used and studied, these properties are becoming increasingly apparent and important in its use in medical devices.

The practical application of HA is reported in following tissue engineering areas:

- vascular tissue engineering;
- cartilage tissue engineering;
- bone tissue engineering; and
- skin and soft tissue engineering

Hyaluronic acid is used for the encapsulation of all those cell lines whose extracellular matrix is rich in glycosaminoglycans or hyaluronic acid itself. For example, hyaluronic acid has been studied extensively for cartilage tissue engineering, demonstrating its ability to support chondrogenic differentiation of mesenchymal stem cells and the formation of cartilaginous matrix. [26] Another important field of application of this polymer is neural tissue engineering

since hyaluronic acid has an important role in the development of the central neural system, in nerve regeneration, in astrocyte activation and proliferation after a spinal cord injury. [27]

Hyaluronic acid is biodegradable in mammals. It is rapidly degraded by hyaluronidase, ß-glucuronidase and ß-N-acetyl-glucosaminidase up to the formation of low molecular weight hyaluronic acid and oligosaccharides that enter the glycolytic pathway. Being naturally present in most connective tissue, hyaluronic acid is commonly used for the encapsulation of cells whose extracellular matrix is rich in hyaluronic acid such as chondrocytes. [24]

Pectin

Pectin is an essentially linear anionic polysaccharide present in the cell wall of various plants, which has been widely used in food and pharmaceutical industries as a gelling, stabilizing and/or encapsulating agent. Like most other plant polysaccharides, it is both polydisperse and polymolecular and its composition varies with the source and the conditions applied during isolation. It is well known that gelation occurs when pectin is in contact with divalent cations. This gelling property has made pectin a suitable delivery system to escort active compounds from the mouth to the colon. It may be used to create various dosage forms with specific drug release profiles.

The composition and structure of pectin are still not completely understood although pectin was discovered over 200 years ago. The structure of pectin is very difficult to determine because pectin can change during isolation from plants, storage, and processing of plant material. At present, pectin is thought to consist mainly of D-galacturonic acid (GalA) units , joined in chains by means of a-(1-4) glycosidic linkage. These uronic acids have carboxyl groups, some of which are naturally present as methyl esters and others which are commercially treated with ammonia to produce carboxamide groups (Figure 8). [38]

Depending on the number of methyl ester groups (referred to as the degree of esterification, DE) pectin is classified as high methoxy pectin (HMP) (DE > 50%) or low methoxy pectin (LMP) (DE < 50%). The affinity of LMP towards divalent cations increases when decreasing DE or ionic strength, and when increasing the polymer concentration. Moreover, the distribution of the galacturonate residues has a strong effect on the polymer's ability to bind various divalent cations. [22]

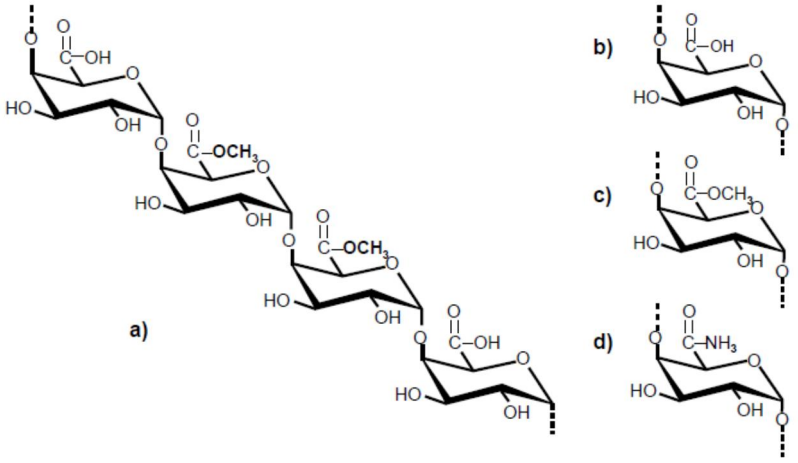

Fig. 8: (a) A repeating segment of pectin molecule and functional groups: (b) carboxyl; (c) ester; (d) amide in pectin chain [38]

Carbohydrate polymers, such as pectins, are generally considered the most effective regarding colon targeted selectivity. In fact, pectins are degrated by colonic microflora in spite of their resistance to the enzymes present in the stomach and intestine. Moreover, these biodegradable natural carbohydrates appear of great and practical interest due to low cost and wide availability. However, pectins are not efficient in retaining drug during transit through the upper gastrointestinal tract, due to their solubility and swelling properties in aqueous media.

Gelling properties of pectins are mainly due to the carboxyl groups able to engage in coordination bonds with divalent cations forming an ñegg-boxò structure. However, the type of pectin and its concentration, as well as the cross-linking conditions are key parameters strongly affecting gelled microparticles performance. [21]

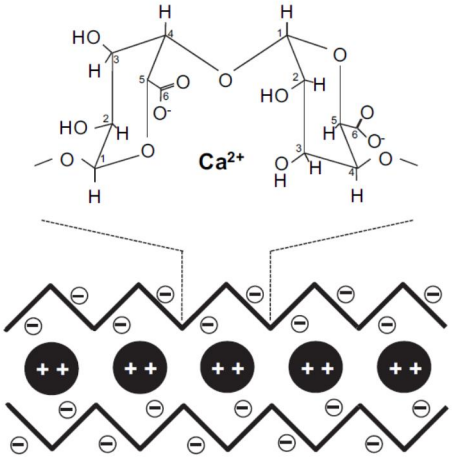

Fig. 9: Schematic representation of the ógegg-boxô model for junction zone formation in pectin-calcium gels. Polygalacturonic acid chains are represented by black lines, calcium ions and carboxyl groups by circles [23]

Dissolved high methoxylated pectin (HM pectin) can form a gel in acidic medium (pH < 3.5) in presence of a cosolute, typically sucrose at a concentration above 55% (w/w). Such gels are often referred to as ógacid gelsô. The high sugar concentration results in a decreased water activity, promoting hydrophobic interactions between methoxyl groups.

The low pH is required to reduce dissociation of carboxyl groups, hence to diminish electrostatic repulsion. These non-dissociated carboxyl groups can form hydrogen bonds with secondary alcohol groups.

Dissolved low methoxylated pectin (LM pectin) can form a gel in presence of calcium ions or other divalent metal ions. In this case, ionic linkages are formed via calcium bridges between dissociated carboxyl groups . This type of gel is often denoted as a ócalcium gelô [23]

A, Interaction through insertion of Ca^{2-} ions between the unesterified carboxyl groups of the galacturonosyl residues of two HG (homogalacturonan) chains. More than nine contiguous calcium bridges are required to generate a stable connection.

B, HG molecules can also be cross-linked by borate-diol esters, which can be formed between the apiofuranosyl residues of the 2-*O*-methyl-D-Xylcontaining side chains.

C, It has been suggested that uronyl esters can be formed as a result of a transesterification reaction, in which a methyl-esterified GalA residue is the donor substrate. In principle, any wall polysaccharide can serve as an acceptor substrate (Figure 10). [39]

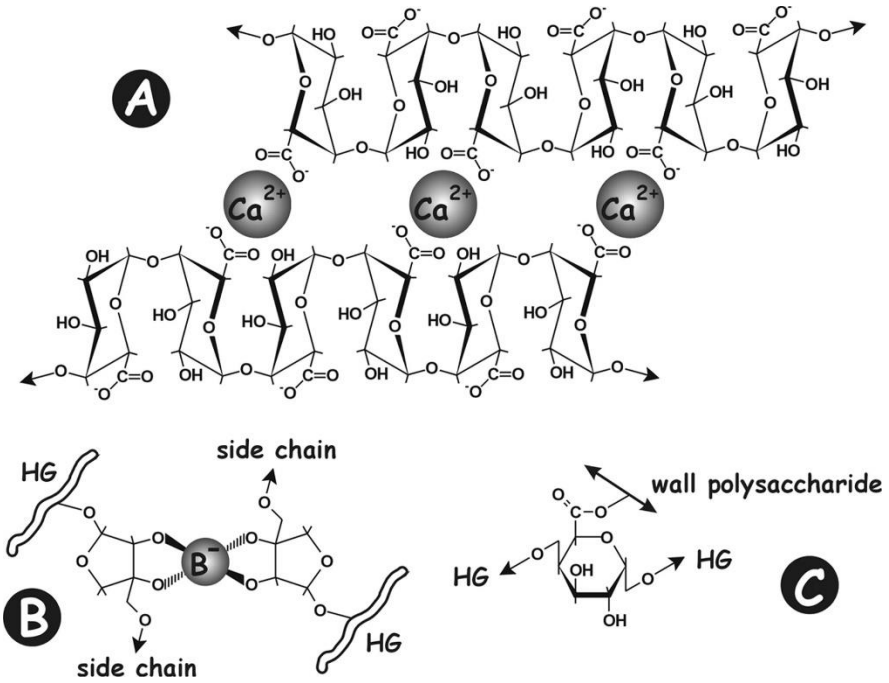

Fig.10: Pectin molecules cross-linked in different ways. [39]

Limitation:

Pectin cannot be used with electrostatic devices. In that case the laminar jet-break-up technology is recommended.

Carrageenan

Carrageenan is a water-soluble anionic polysaccharide derived from the *Rhodophyceae* red algae by alkali extraction. Carrageenan is a galactan, like agarose, and it consists of repeat sequences of β-D-galactose and α-D-galactose with variable proportions of sulfate groups. In carrageenan, the β-galactose is D while in agarose it is L. Commercially available carrageenan can be divided into three families based on the position and number of sulfate groups: κ-(kappa), ι(iota) and λ-(lambda) carrageenan carrying 1, 2 and 3 sulfate groups, respectively.

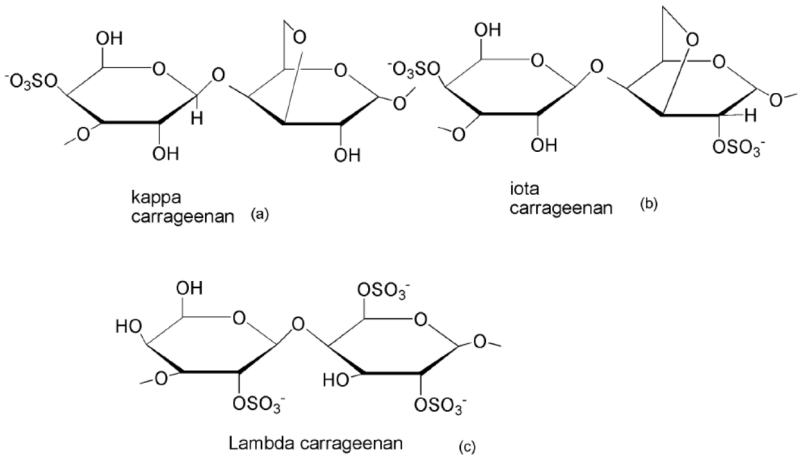

Fig. 11**:** Chemical structure of three types carragennan [28]

Aqueous solutions of ə- and ɘcarrageenan can reversibly form hydrogels in the presence of cations, while ə-carrageenan does not undergo a solï gel transition. In fact, carrageenan in solution has a random-coil conformation and upon cooling ə- and ɘconformation becomes a double helix with the sulfate groups pointing outwards, while the higher sulfate content of ə-carrageenan inhibits the formation of the helicoidal structure. [29] The positive cations in solution neutralize the charge of the sulfate groups allowing a tighter aggregation of the helices. Divalent cations are effective in promoting the formation of strong hydrogels for ə- and ɘcarrageenan while monovalent ions are particularly effective on ə-carrageenan. Hydrogels prepared using ɘcarrageenan are softer and more deformable than those prepared using ə-carrageenan. [24]

Despite the extensive and documented use of this polymer as an inducer of chronic and acute inflammation, there is no consensus on its effect on the inflammatory response of the host when newly developed purified carrageenan is used.

Also, both ɘ and ə-carrageenan hydrogels have been used for cell encapsulation by ionic cross-linking and by the formation of complexes with polycations such as chitosan.

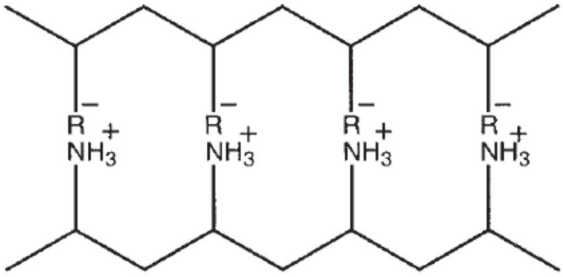

Fig. 12: A schematic depiction of the polyelectrolyte complex between chitosan and biopolymer; R OSO_3^- (kappa-carrageenan) [54]

Interaction by iota-carrageenan with divalent cations, such as Ca^{2-} ions, are considered to be more effective than with monovalent cations. In order to understand this behavior a schematic representation of iota-carrageenan double helix structure, proposed by Tako et al., is presented in Figure 13. According to this model, intramolecular bridging with Ca^{2-} may take place between the sulfate groups of adjacent anhydro-D-galactose and D-galactose residues. [40]

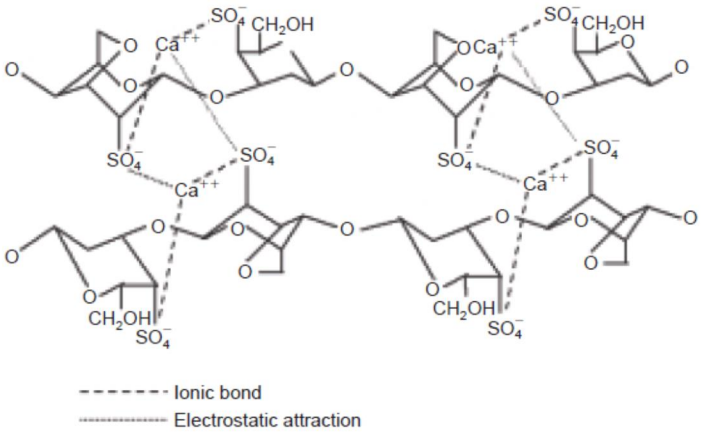

Fig. 13: Schematic representation of Ca-bridges between iota-carrageenan molecules [40]

Similar to alginate the degradation of carrageenan hydrogels is driven by the exchange of ions with the surrounding fluids, while only some bacteria produce the enzymes that can cleave the polymer chain. [24]

Chitosan

The biopolymer is characterized as either chitin or chitosan according to the degree of deacetylation (DD) which is determined by the proportion of D-glucosamine and N-acetyl- D-glucosamine. Structurally, chitosan is a straight-chain copolymer composed of D-glucosamine and N-acetyl-D-glucosamine being obtained by the partial deacetylation of chitin. Chitosan is the most abundant basic biopolymer and is structurally similar to cellulose, which is composed of only one monomer of glucose (Fig. 14). Chitosan solubility, biodegradability, reactivity, and adsorption of many substrates depend on the amount of protonated amino groups in the polymeric chain, therefore on the proportion of acetylated and non-acetylated D-glucosamine units. The amino groups (pKa from 6.2 to 7.0) are completely protonated in acids with pKa smaller than 6.2 making chitosan soluble. Chitosan is insoluble in water, organic solvents and aqueous bases and it is soluble after stirring in acids such as acetic, nitric, hydrochloric, perchloric and phosphoric. [17]

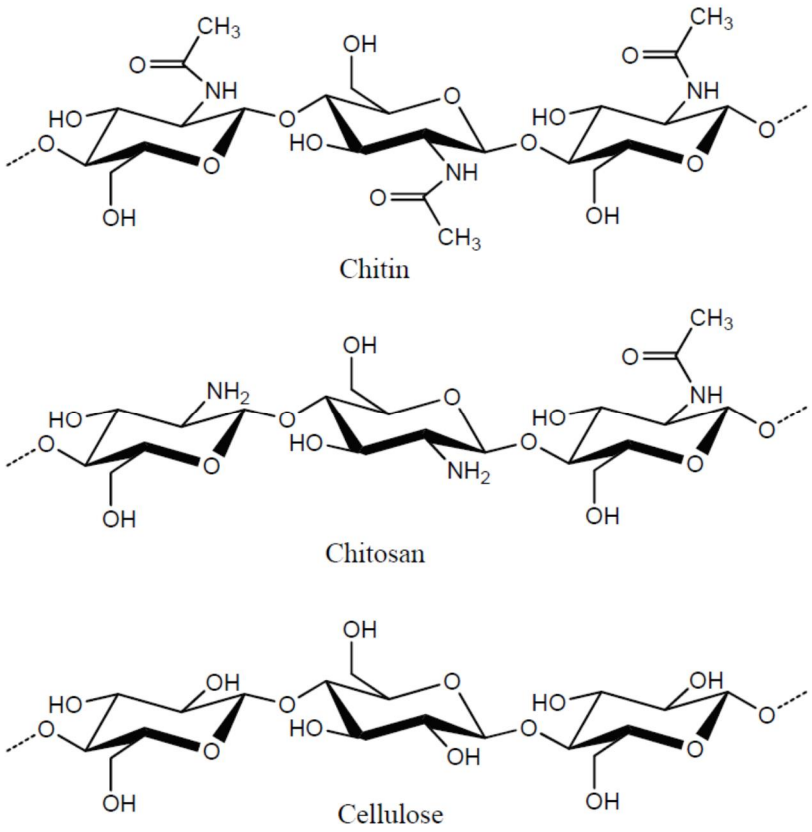

Fig. 14: Structures of chitin, chitosan, and cellulose

By the chitosan gel preparation, the chitosan is dissolved in an acetic acid solution (pH = 4) The prepared solution is injected drop by drop using a syringe in a gelling solution (solution of sodium hydroxide 3M or sodium dodecylsulphate solution 50mM). The obtained solution is maintained at room temperature (25°C) and the obtained hydrogel (in the form of balls) is filtered at the end.

The addition of NaCl in the $CaCl_2$ can influence alginate to ñstickò better to the chitosan polymer.

In addition, research has shown that the addition of monovalent salts such as NaCl can have an obvious effect on the chitosan crosslinking or PEC formation processes.

For non-reactive salts added into polymeric aqueous solutions, their effects can be generally described as: (1) osmotic pressure, (2) ñsalting outò and (3) screening effects.

(1) **Osmotic pressure**. For hydrogels formed with polyelectrolytes such as chitosan, there is usually a difference in mobile ion concentrations into and out of gel matrices, which is caused by macroscopic electroneutrality requirements. This results in an osmotic pressure between the gel matrix and the surrounding solution. The minimisation of this osmotic pressure is generally achieved via gel swelling (dilution of the network charge density). Moreover, the charges on the polymer chains will generate electrostatic repulsion forces which facilitate the gel swelling process. When salt is added into the system, the difference between ion concentrations inside and outside of the gel matrix is reduced. Consequently, the swelling force decreases with increasing salt concentration. Therefore, the addition of salts into gel systems will affect the gel particle size.

(2) **ñSalting outò.** The ñsalting outò effect is mainly discussed with non-ionic polymers. When salts are dissolved in polymer solutions, they can modulate polymerï water interactions by competing for water molecules in the polymer hydration sheath, which results in the clustering of hydrophobically- substituted regions.

(3) **Screening effects.** The electrostatic charge screening effect is a well-known salt effect in aqueous solutions. Typically, a positive ion in aqueous solution always has a higher density of negative ions surrounding it and its electric field becomes screened. The addition of NaCl into the chitosanï NaCS (sodium cellulose sulfate) system may affect the interaction between chitosan and NaCS because of the screening of chitosan positive charges by Cl, as salt ions may move more freely and faster than the polymeric NaCS in aqueous solutions. This salt-induced screening effect may also result in reduced electrostatic repulsion between the charged groups on the chitosan polymer chains and thus decrease the gel swelling trends. [41]

Use of the Chitosan and Alginate in Microencapsulation

The use of new natural polymers as drug carriers has received considerable attention in the last few years. One of the goals of such systems is to prolong the residence time of a drug carrier in the gastrointestinal (GI) tract. [42,43] The bioadhesive bond can be of a covalent, electrostatic, hydrophobic, or hydrogen bond nature. [44] Ionic polymers have been reported to be promising for bioadhesive medical applications, and increased charge density will also give better adhesion, this suggests that electrostatic interactions are of great importance. [44]

There are different approaches of the use of alginate and chitosan in the encapsulation:

1. Creation of the alginate microcapsules and its later exposure to chitosan solution (Two stage procedure);

2. Creation of the alginate microcapsules with their hardening in the solution containing chitosan (One stage procedure);

Creation of the alginate microcapsules and its later exposure to chitosan solution

(Two stage procedure)

The beads are made from alginate as the core and chitosan as the outer coating. The developed beads can be consisted of an active component entrapped within sodium alginate and coated with chitosan as an outer layer to control the release of the active component. Alginate-chitosan beads are prepared by the ionotropic hydrogelation method with a polyelectrolyte complex reaction between two oppositely charged polyions with sodium alginate as the gel core. [51,45,46]

Creation of the alginate microcapsules with their hardening in the solution containing chitosan

(One stage procedure)

In this method the active component (drug, amino acid, peptide, or living cell) are suspended sodium alginate solution (1.2% (w/v)). The suspension is afterwards sprayed through a nozzle located inside the head of a droplet-forming apparatus into chitosan solution where they were allowed to gel for 20 or 30 min. The chitosan solutions is prepared in (N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid])ï buffered calcium chloride (13 mM HEPES, 1.5% (w/v) $CaCl_2$, pH 6 or 6.3; Sigma Aldrich). Alginate-chitosan microcapsules are then washed three times in HEPES and can be used in the further experiments. [46]

Another approach is to prepare sodium alginate solution (1.5% (w/v) containing 0.01*M* NaCl and active component and to extrude it through a nozzle. The droplets from the nozzle are pulled off into a 0.5% chitosan solution containing 0.01*M* NaCl and 0.05*M* calcium chloride whose pH is adjusted to an appropriate value. The droplets are then allowed to harden for 30 minutes in the hardening bath. After hardening, the microcapsules are rinsed with distilled water and transferred into saline for the further examination. [47]

The sodium alginate solution containing hemoglobin as active component was dropletized trough a nozzle into a 0.1% HCl chitosan solution (4 or 8 g/L) containing *0.05M* $CaCl_2$ and whose pH was adjusted at a given value with 1*M* NaOH (treatment I). In the second method, the alginate droplets were pulled off in a chitosan solution (in 0.1% HCl) whose pH was adjusted as described above and the capsules thus obtained were isolated, then treated with *0.05M* $CaCl_2$ (treatment II). [48]

In another study a chitosan solution of various concentrations (100 mL) containing 1.5 g% $CaCl_2$ was used for gellation of alginate containing insulin. A series of chitosan solution in 0.01M HCl (0.1, 0.3, and 0.5%) was prepared and the pH was adjusted to 5.7 using 0.1N NaOH solution, and finally filtered to get a clear solution. The rinsed capsules are allowed to dry in air at room temperature until constant weight is achieved. [49]

Creation of the glucan particle alginate/chitosan microcapsules

Glucan particles (GPs) are 2ï 4 εm spherical, hollow, porous shells extracted from Bakerôs yeast, *Saccharomyces cerevisiae.* The surface of the GPs is composed primarily of 1,3-ɒ-glucan and the particles are efficiently phagocytosed via receptor-mediated cell uptake by macrophages, phagocytic cells expressing glucan receptors. The hollow cavity of the GPs allows for efficient absorption and encapsulation of payload molecules. Rifampicin (Rif), a drug used in tuberculosis treatment, was encapsulated as active component by precipitation in GPs and trapped using a calcium alginate or chitosan hydrogel to seal the pores of GPs and slow Rif release. [10]

Collagen and Gelatin

Collagen is the most abundant protein in humans and the main component of the extracellular matrix of many tissues. It is mostly synthesized by fibroblasts and osteoblasts. There are 29 different forms of collagen in the body, the most ubiquitous which is type I collagen, which comprises triple Ŭ-helices, which in the correct environmental conditions self assemble to form a fibrillar structure. [14]

Collagen proteins consist of a unique triple helix extending over a large portion of the molecule. The helices assemble in complex supramolecular structures. Every third amino acid of the chain of the helix is glycine. Glycine, a very small amino acid, occupies the centre of the helix allowing a tight packing of the three chains. About one-third of the remaining amino acids are proline and hydroxyproline and have their side chains pointing outwards from the helix. [32] Collagen type I is typically dissolved in diluted acid. Collagen self-assembles to form a hydrogel when the solution is neutralized (e.g. with NaOH) and heated to physiological temperature. [24]

For cell encapsulation, cells can be mixed with the neutralized collagen solution and the suspension can then be moved to an incubator. Collagen contains some adhesion motifs as RGD (Arg-Gly-Asp) an important tripeptide for the interaction between a variety of cells and the extracellular matrix. Collagen can be rapidly biodegraded in mammals via collagenases and metalloproteinases to yield the corresponding amino acids. The rate of degradation can be controlled by enzymatic treatment or chemical cross-linking.

Because collagen is the main component of the extracellular matrix of many tissues, and its self-assembling tendencies [14], it has found many potential applications for tissue engineering (cartilage repair, mesenchymal stem cell-based therapies, bone regeneration, etc.) Collagen is the most common clinically used biomaterial for skin repair [24]. Rat tail collagen, for example, may be dispersed in acid medium and when neutralised in culture forms gels which have been widely used since they allow cell adhesion. [14]

The two major limitations of collagen-based scaffolds are their weak nature and their extensive contraction by encapsulated cells. [14]

The gelatin most important substitutes are agar agar, carrageenan, Vegan Gel powder (made of vegetable gum, adipic acid, tapioca dextrin, calcium phosphate and potassium citrate) and pectin. These materials are prion free materials.

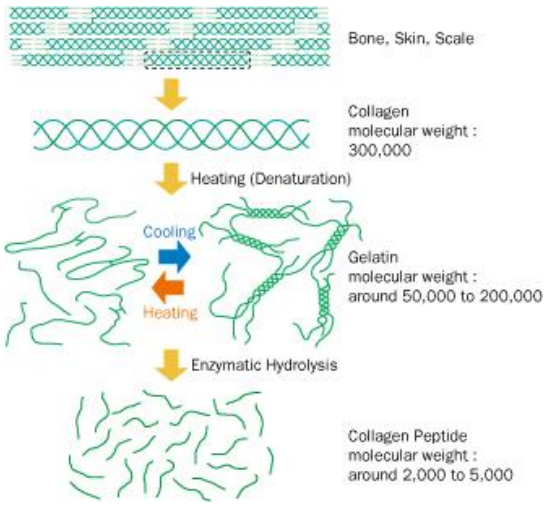

Fig. 15: Transformation of collagen to gelatin [55]

The triple helix of collagen can be broken down into single strain molecules to obtain gelatin. Two different types of gelatin can be obtained by treating collagen: one is the result of the hydrolysis of the amide groups of asparagine and glutamine into carboxyl groups, while the other is the result of an acid treatment. The carboxyl groups make gelatin negatively charged.

Gelatin is thermo-responsive, undergoing a reversible solï gel transition by cooling a water-based solution of the polymer below 35°C. The hydrogel can be liquefied by heating it to physiological temperature. This property has been exploited to fabricate hydrogels with an inner gelatin core that ómeltsô once placed in physiological conditions [34] or to fabricate porous cell-laden scaffolds with the gelatin beads acting as porogen. [33]

Gelatin can be chemically modified to encapsulate cells so that it does not liquefy when placed at physiological temperature. For example, methacrylate groups can be added to the side chains of gelatin, resulting in photo-cross-linkable gelatinï methacrylamide. [35]

Gelatin is widely used in pharmaceutical and medical applications due to its biodegradable nature. [14]

Molecular weight of gelatin is 30 to 200kDa. Gelatin has the property of swelling in water, taking up water 5 - 10 times of its own weight. At temperatures above 50°C, gelatin liquefies. Its solidification point is around 25°C. In the pharmaceutical industry gelatin is used for generation of hard and soft capsules.

Fig. 16: Typical setup for microencapsulation with gelatin
(VARD2Go ï Aerodynamic Thermal Option)

Example - Gelatin coating of alginate microcarriers

Alginate microcarriers were incubated in gelatin 1% (w/v) solution for 2h with cycles of 2 min at 750 rpm and 10 min with non-agitation at 37°C. The supernatant was discarded and a aquous solution of glutaraldehyde 0.4% (v/v) was added to promote covalent crosslinking of

gelatin. The crosslinking time was 30min at 750 rpm, 22°C continuausly. The gluteraldehyde solution was removed and the crosslinked gelatin coated alginate microcarriers were incubated in a glycine solution (100 mg/mL) for 1 hour at room temperature with adequate agitation. Quenched with glycine solution was removed and the microcarriers were washed twice with PBS (phosphate-buffered saline). The microcarriers were observed in the optical microscope for diameter distribution analysis. [52]

Fibrin

Fibrin is a natural fibrous protein involved in the clotting of blood. It is polymerized from fibrinogen and thrombin into a mesh structure that can form a hemostatic clot (in conjunction with platelets) over a wound site.

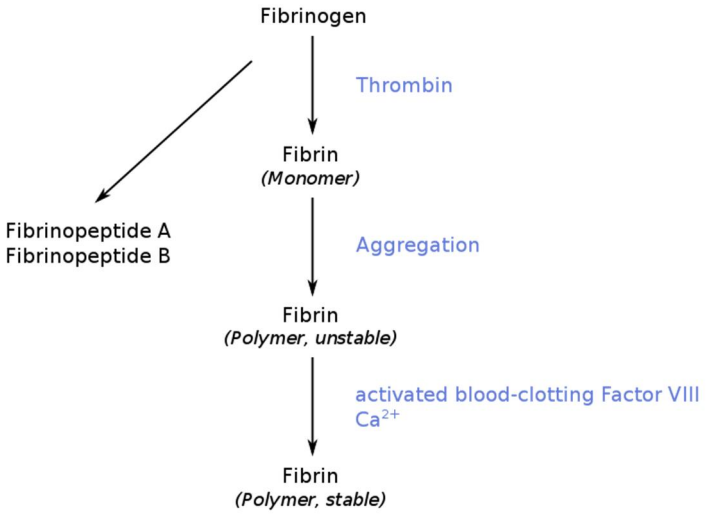

Fig. 17: Genesis of fibrin out of fibrogen

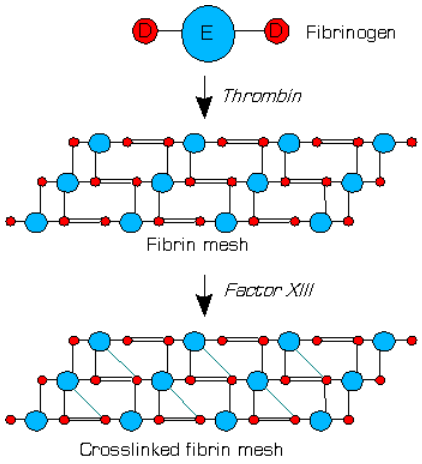

Fig. 18: Cross-linking by thrombin and stabilization by activated factor XIII

Fibrin is a biopolymer of the monomer fibrinogen. The fibrinogen molecule is composed of two sets of three polypeptide chains named AŭŬ, Bƀ, and ɔ, which are joined together by six disulfide bridges. Fibrin is formed after thrombin-mediated cleavage of fibrinopeptide A from the AŭŬ chains and fibrinopeptide B from the Bƀ chains, with subsequent conformational changes and exposure of polymerization sites. This generates the fibrin monomer that has a great tendency to self-associate and form insoluble fibrin. Further, the blood coagulation factor XIIIa is a transglutaminase that rapidly cross-links ɔ chains in the fibrin polymer by

introducing intermolecular ε-(γ-glutamyl) lysine covalent bonds between the lysine of one γ-chain and glutamine of the other. This covalent cross-linking produces a stable fibrin network that is resistant to protease degradation. This effect can be reinforced by introducing chemical cross-linker such as genipin. [20]

Fibrin scaffolds can be engineered as a tissue substitute that is biocompatible and biodegradable. Proliferation and differentiation of the stem cells can be achieved in a fibrin matrix, and fibrin alone or in combination with other materials has been used as scaffolds to regenerate adipose tissue, bone, cartilage, etc. The fibrin microbeads can be produced in hot oil, and the cells can be seeded on the surface of the beads, but in that case the cells are not encapsulated inside the beads. It is difficult to construct cell-encapsulated fibrin microbeads due to the sticky nature of fibrin. There were attempts to fabricate cell-encapsulating fibrin beads of about 3 mm in diameter, by first preparing an alginate-fibrin mixture and then extracting the alginate. However, these large beads, when mixed into a paste such as calcium phosphate cement, would not be injectable because the beads are larger than the needle size.

There were reported the stem cell-encapsulating alginate-fibrin microbeads with diameters of several hundred μm, which can degrade rapidly and release the cells in a few days. These fine microbeads would be injectable with a calcium phosphate cement or another paste such as an injectable polymer. Once delivered, these microbeads could degrade inside the scaffold to release the cells and create macropores of several hundred mm in size, which are suitable for cell migration and tissue ingrowth. In addition, the influx of nutrients and the outflux of biological metabolites produced by the encapsulated cells would be easier due to the small size and high surface area of the smaller microbeads. [19]

A totally synthetic fibrin glue has been developed. The molecular structure is a copolymer composed of *N*-isopropyl acrylamide (NIPAM) units and vinyl monomer units which has a cell-adhesion peptidyl moiety (Arg-Gly-Asp- in the side chain. The buffer sollution is viscous and transparent at room temperature, but on elevation to physiological temperature, sponteneous precipitation occurs owing to a thermoresponsive phase transition derived from the NIPAM unit. On mixing with platelet-rich plasma, the peptidyl moieties bound to platelet receptors. When applied to living tissues, spontaneous precipitation and subsequent platelet aggregation occurred, indicating that the synthetic bioactive polymer effectively functions like fibrin glue. This indicates that the water-soluble copolymer developed here, which incorporates thermoresponsiveness and cell adhesivity, biomimics fibrin glue. [53]

2. Extrusion Techniques - Microgel Encapsulation

The main principle behind all techniques involves extrusion of a liquid mixture of an encapsulating material and bioactive substance through an orifice and formation of droplets at the discharge point of the nozzle. Different mechanisms of droplet formation arise due to the interaction of gravitational, surface tension, impulse, and frictional forces [6,7], according to which extrusion techniques are classified into simple dripping, electrostatic extrusion, coaxial airflow, vibrating jet/nozzle, jet cutting, and spinning disk atomization. The droplets formed are immediately solidified to capsules by either physical (e.g., cooling or heating), or chemical process (e.g., gelation).

Principle of Drop Generation by Gravity

Simple dripping applies low liquid velocity, where the extruded liquid sticks to the edge of the nozzle until the gravitational force is high enough to overcome the surface tension, resulting in the release of a drop. Increased liquid velocity amplifies droplet formation and can result in coalescence of the droplets and, consequently, reduced monodispersity. The main factor which determines the size of the droplet is an orifice diameter [8]. The main drawbacks of this technique, such as very low quantities of droplets produced (insufficient for industrial application) and droplets having very large diameters (>2mm).

Fig. 19: Head for 30 nozzles and customised nozzle ï Nisco Unit **VARGravity2Go10**

Principle of Electrostatically Assisted Spraying

The principle is based on using an electrostatic potential to pull droplets from a needle top into a gelling bath. A voltage is applied between the needle feeding the hydrogel solution and an electro conductive solution underneath. This technique refers to electrostatic extrusion also known as electro-spraying.

The voltage forces the droplets to fall off the needle tip before it has grown to the point were it falls off due to its own weight. The beads are formed when droplets fall into the hardening solution.

The applied high voltage (0 ï 30kV) has been confirmed not to damage the encapsulated cells and proteins. Electrostatic extrusion technique allows production of very small particles, with uniform size distribution.

The variance of this technique is electrospinning which is based on high-voltage spinning of a polymer solution to produce (nano)fibers/(nano)webs.

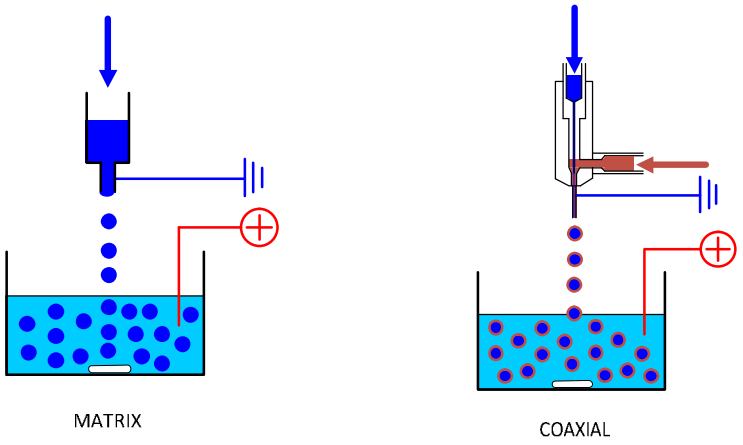

Fig. 20: Principle of electrostatically assisted spraying

Principle of Coaxial Airflow Induced Dripping

In coaxial airflow technique, a stream of compressed air is used to pull the liquid droplets from the nozzle at a faster rate compared to the normal gravitational force [9]. This technique allows production of microcapsules/beads with diameters >200ε m, of uniform size and shape, under reproducible and mild conditions and can be performed under sterile conditions. The main disadvantage of the coaxial airflow technique is low production rate.

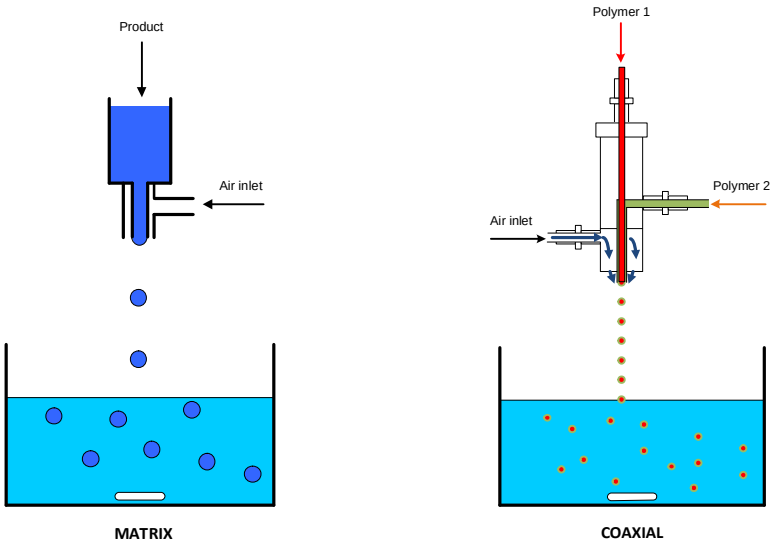

Fig. 21: Principle of coaxial airflow bead generator

Principle of Aerodynamically Assisted Jetting

The AAJ (Aerodynamically Assisted Jetting) phenomenon takes place within a pressured chamber containing a needle accommodating the controlled flow of media. The exit orifice is placed centrally and in line with the needle exits. The input of a controlled pressure into the chamber gives rise to a pressure gradient across the exit orifice and generates an aerodynamic flow field. This developed flow field provides the driving mechanism for drawing out media emerging from the needle through the exit orifice.

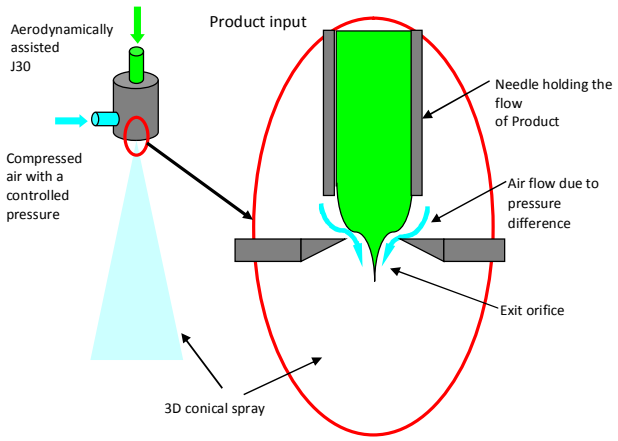

Fig. 22: Principle of aerodynamically assisted jetting

Near homogenous, very small particles around 20 micrometre in diameter, with a minimised danger of clogging are produced by AAJ.

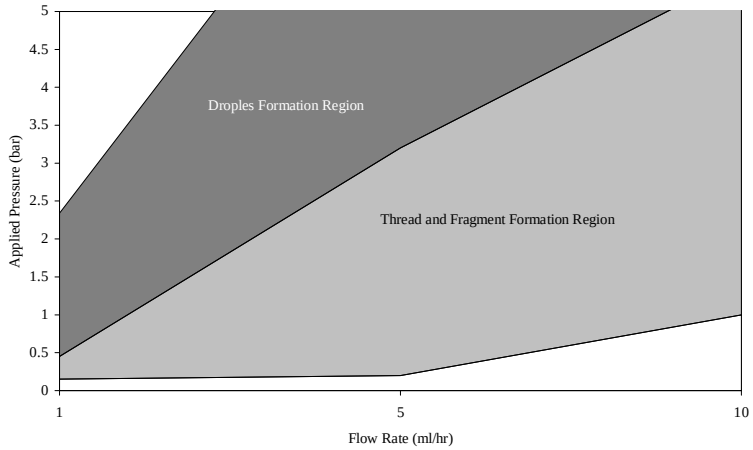

Fig. 23: Operational map for an aerodynamically assisted jetting device [13]

It is very important to create an operational map for each specific microencapsulation process and medium that gives rise to the formation of a two phase operational guide having defined regions where droplets and threads are formed. The line demarking a fence in between these two regions is seen to vary slightly but overall the variation is insignificant. [13].

Principle of Laminar Jet Breakup

Higher production capacity can be achieved with use of controllable liquid jet breakup. This approach is utilized in vibrating jet / nozzle technique, jet cutting technique, and rotating (spinning) disk atomization. These techniques differ only whether jet breakup is accomplished by vibrating a nozzle with a frequency of defined amplitude, or by cutting wire, or by a rotating disk, respectively. [7]

Vibrating Jet / Nozzle Technique

The electromagnetic laminar jet breakup technology is based on the principle that a laminar jet of a liquid feed passing a nozzle of appropriate diameter is broken into equally sized droplets by a superimposed mechanical vibration. The vibration has to be done in resonance of the Plateauï Rayleigh instability and leads to very uniform droplets.

The Plateauï Rayleigh instability is named for Joseph Plateau and Lord Rayleigh who in 1873 found experimentally that a vertically falling stream of water will break up into drops if its length (so called ñwave length of disturbanceò in specialist literature) is greater than about 3.13 to 3.18 times its diameter.

Using the natural liquid instability and replacing the natural irregular disturbances with the regular permanent mechanical vibration, small uniform droplets are produced. The droplets are further processed in order to form particles by crosslinking or polymerization or other hardening process of the liquid feed.

The particle diameter can be predicted and controlled by choosing the nozzle diameter, volumetric flow and superimposed frequency.

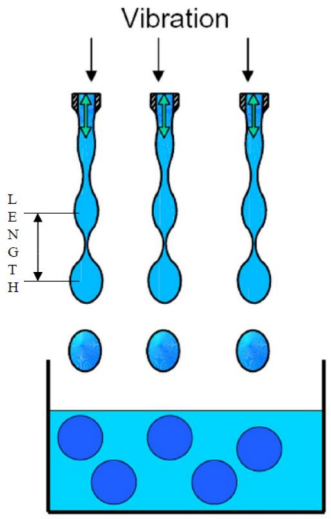

Fig. 24: Principle of vibrating jet/nozzle technique

Jet Cutting Technique

For bead production by jet cutting, the fluid is pressed with a high velocity out of a nozzle as a solid jet. Directly underneath the nozzle the jet is cut into cylindrical segments by a rotating cutting tool made of small wires fixed in a holder. Driven by the surface tension, the cut cylindrical segments form spherical beads while falling further down to an area where they finally can be gathered.

Bead generation with the jet cutting principle is based on the mechanical impact of the cutting wire on the liquid jet. This impact leads to the cut together with a cutting loss, which in a first approach can be regarded as a cylindrical segment with the height of the diameter of the cutting wire. This segment is pushed out of the jet and slung aside where it can be gathered and recycled.

As only a mechanical cut and the subsequent bead shaping driven by the surface tension are responsible for bead generation, the viscosity of the fluid has no direct influence on the bead formation itself. Thus, the jet cutting technology is capable of processing polymers with viscosities up to several thousand mPas (for example: alginate, chitosan, pectinate, carrageenan cellulose derivate (CMC, SEC, CS) and polyvinyl alcohol (PVA)), which can be processed with application of other microencapsulation technologies.

The size of the beads can be adjusted within a range of between approx. 200µm up to several millimetres.

The main parameters of this principle are the nozzle diameter, the flow rate through the nozzle, the number of cutting wires and the rotation speed of the cutting tool.

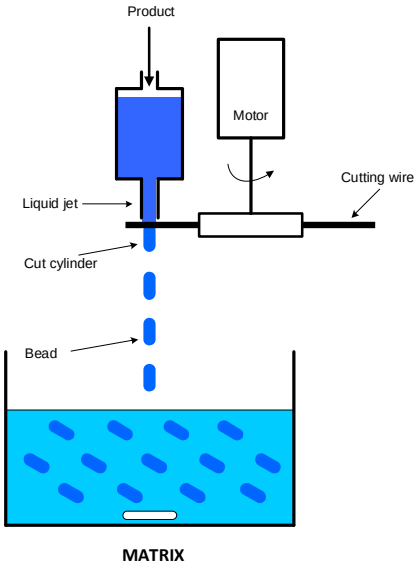

Fig. 25: Principle of jet cutting technique

Rotating (Spinning) Disk Atomization

Spinning disk atomization is a technique which is based on the disintegrating of a feed liquid performed on disc(s) to produce droplets [10]. When a liquid is dropped onto the surface of a rotating disk it is centrifugally accelerated to a high velocity and distributes as a thin film on the disc. Depending on the flow rate of the feed, droplets are then released due to the centrifugal forces at the tip (teeth) of the rotating disk or from ligamentry streams released from the edge of the disk. The size of the droplets produced is determined mainly by the rotation speed of the disk. This simplistic technique has shown the capability to produce microspheres from 200 µm in diameter, with a narrow size distribution and is easily scalable, with possible production capacities of tons/ day using a multi-disk system. [11]

Principle of Electrospinning

Electrospinning is a notable thread-fabrication technique used to produce non-woven fibrous materials with thread diameters in the order of a few nanometers to >1εm. The main application field of electrospinning are tissue engineering, medical devices (cardiovascular, dental and fiber bundle implants, nerve regeneration, wound dressing) and drug delivery.

The main principle of the electrospinning process is the effect that high voltage has on a polymer solution.

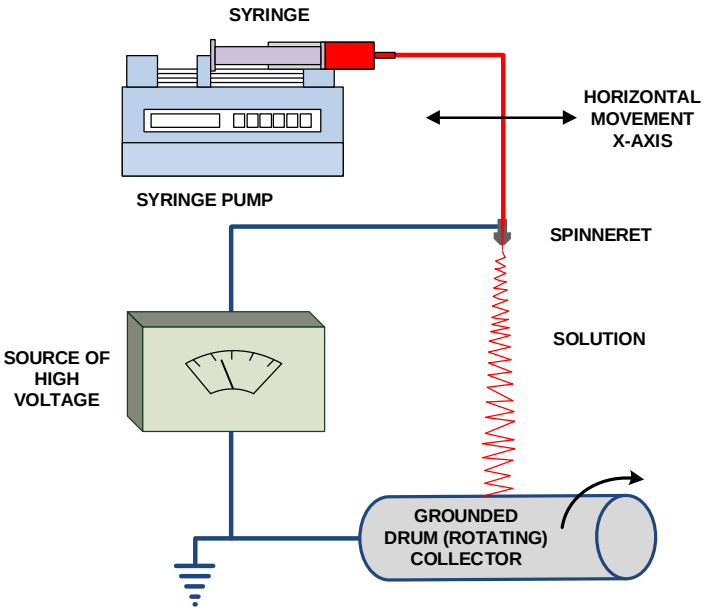

Fig. 26: Schematic illustration of an electrospinning unit with rotating drum collector

High voltage (tens of kV) is connected to the end of a capillary containing liquid solution. Once the electric field intensity increases, the hemispherical surface of the liquid at the end of the capillary extends and creates a conical shape known as a Taylor cone. By increasing the electric field further, the critical value at which the repulsive electrostatic force overcomes the surface tension is reached, and the charged strand squirts out of the end of the Taylor cone. The strand of polymer solution undergoes a process of instability and lengthening, during which solvent evaporates. The fibers are then deposited on the collector. The resulting product is a non-woven fibrous layer.

In the classical electrospinning arrangement, nanofibers are deposited on the collector at random. For some applications, however, an ordered structure is more appropriate. The method used to organize the nanofibers can be influenced by the type of collector.

The following substances can be used in the electrospinning: dimethylformamide (DMF), polyacrylonitrile (PAN), polycaprolactone (PCL), polyethylene glycol (PEG) and poly(lactic-*co*-glycolic acid) (PLGA).

3. Advantages and Limitations of Hydrogel Encapsulation

Advantages

Hydrophilic compounds have been mainly entrapped into gel particles, creating matrix type of encapsulates. Bacteria, yeast cells, and enzymes have been extensively encapsulated within gel particles for obtaining so-called immobilized biocatalysts that can be used for bioprocess intensification (e.g., fermentation processes).

The microgels are suitable formulations for hydrophobic compounds. The lipophilic compounds such as oils can be processed by co-extrusion. A device is consisting of a double capillary, with the core (containing an oil compound) in the inner capillary and the shell in the outer capillary. In this way, beads are generated with the internal substances (the core) being completely surrounded by a physical barrier (the shell). [12]

The common advantage of all extrusion techniques is that they do not involve severe conditions, both in terms of temperature and solvents.

Another important issue is possibility to process viscous polymer solutions. In order to challenge the use of high viscous polymer solutions as encapsulating materials, some improvements have been made, based on the fact that an increase in temperature reduces the viscosity of the polymer solution. Thus, the apparatus has been developed for heating and/or maintaining polymers solutions at controllable temperatures as they pass through the pulsation chamber, before extrusion and breakup. This apparatus, can supply a controllable temperature to the chamber that subsequently heats and/or maintains the temperature of the polymer during extrusion and jet breakup. Use of such device should enable encapsulation into viscous materials like highly concentrated biopolymer solutions, gelatins, and gums.

Among the ingredients that can be used to produce microgels, the polysaccharides are particularly attractive, mainly due to their technological properties and because they are generally recognized as safe (GRAS) such as alginate, chitosan, kappa-carrageenan, and gellan gum. Moreover, although many polymers require organic solvents for their dissolution and subsequent electrospinning, some biopolymers can be electrospun from a watery solution just by adjusting the process parameters and/or changing the solution properties through the addition of proper additives (e.g., whey protein).

In general, the size and shape of microgels produced by any of atomization techniques are influenced by composition, viscosity, and surface tension of a polymer solution, as well as operating parameters characteristic for a given technique. By any of the mentioned techniques, it is possible to produce spherical and uniform microparticles. However, concentration of a polymer solution has to be carefully accorded with processing parameters; otherwise, filaments and irregularly shaped structures may occur.

One promising strategy is to utilize affinity of some biopolymers for polyelectrolyte complexation with oppositely charged polymers. For example, alginate forms important composite materials with plant-derived biopolymers, such as chitosan, carrageenan, and pectin or synthetic polymers, for example, PVA or poly-L-lysine. Chitosan-coated gel beads are the most efficient for retarded release and control of releasing properties during the enteric digestion.

Limitations

The relatively low production rate of more microencapsulation techniques instead of rotating (spinning) disk atomization and laminar jet breakup is a fact. However, the low production rate is not the only limiting factor for a wide application of extrusion techniques in food industry.

One of the important limiting factors of is high viscosity of applied biopolymer. The viscosity of the applied biopolymer can significantly affect the microencapsulation. Since aqueous solutions of polymers can exhibit very high viscosities even at low concentrations, a maximum concentration of the polymer which can be processed is rather low. For example, in the case of sodium alginate, the maximal applicable concentration for matrix encapsulation is approximately 2ï 4 %. In this case the low and high viscosity products are available at the market.

As a consequence of the use of a not suitable biopolymer, sometimes the resulting gel network could exhibit a low density which canô provide the necessary barrier effect in protecting of the encapsulated active ingredient.

Another consequence could be the low encapsulation capacity for water-soluble compounds of small molecular weight since they easily diffuse out into the gelling solution. To prevent this negative effect sometimes is needed to produce multi layer microbeads. For example, the alginate bead can be overcoated with one or more additional chitosan or pectin layes (shells) which can improve the microcapsule characteristics in a specific application.

Some gel particles could have weak mechanical stability and limited chemical stability. They can bi sensitive to pH change and the presence of different monovalent and divalent cations in the solution. Therefore, new strategies to create water-insoluble microparticles, with reduced porosity and retarded release of entrapped substances have to be developed.

Natural biopolymers can be different in their quality and their physical and chemical properties depending on the source from where they are isolated. There are different low, medium and high viscosity biopolymers from different sources available at the market. For example, low viscosity alginate has a very wide range of applications in the microencapsulation technology, but its most important properties can differ form one to the other production batch. Because of that, in the case of the large scale microencapsulation production the blending of different batch products is crucial.

The exponential increase of the dynamic viscosity with the increasing sodium alginate concentration is clearly visible from the following table. Solutions with the sodium alginate content of up to 2 mass % were regarded as low-viscosity solutions and those of 3 mass % and 4 mass % as high-viscosity ones. [9]

Alginate content	Bratislava		Warsaw		Belgrade		Nantes		Brussels[a]	
	η	D	η	D	η	D	η	D	η	D
mass %	mPa s	s^{-1}	mPa s	s^{-1}	mPa s	s^{-1}	mPa s	s^{-1}	mPa s	s^{-1}
0.5	33	100	24	233	–	–	26	300	32	12
1.0	140	10	92	233	–	–	83	300	155	11
2.0	1232	1	667	129	1129	6.3	424	300	1334	1
3.0	5501	0.5	2008	23	5236	2.5	–	–	4632	20
4.0	10516	0.1	10560	10	11251	2.5	–	–	12187	10

a) Measurements carried out at 35 °C.

Table 1: Dynamic viscosity **q** and applied shear rate D for the viscosity measurements of alginate solutions at 30°C in different laboratories [9]

Many hydrogel microcapsules can be very sensitive against drying in the last production stage, as well. In that case, for the applications in products with long shelf lives, hydrogel

encapsulates can be protected with addition of some filler compounds or by some additional overcoating thermoresistant components.

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