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Review

BIODEGRADABILITY OF CHITOSAN BASED PRODUCTS

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ABSTRACT

The biodegradation of chitosan is catalyzed by enzymes or chemicals *in vitro* or *in vivo* and it refers to the breakdown of the polymer substance into smaller fractions such as monomers (D-glucosamine, N-acetyl-glucosamine). When using chitosan in drug-delivery systems and tissue engineering, the biodegradation rate is particularly crucial. The degree of deacetylation (DD) and molecular weight (MW) are the key factors for controlling the biodegradation rates of chitosan. In addition, chemical modifications of chitosan will significantly influence the biodegradation rate. Lysozyme is one of the most common used enzymes for in vitro degradation studies. The study of chitosan degradation *in vivo* can be conducted either by injecting chitosan intravenously or by implanting chitosan subcutaneously in laboratory animals. Oral administration can give valuable results and this technique can be used to study the degradation of chitosan in the gastrointestinal tract. Despite the fact that are numerous studies made so far, the mechanism of chitosan degradation is not yet fully understood.

Keywords: chitosan biodegradation, chitosan derivatives, *in vitro* biocompatibility

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INTRODUCTION

Chitosan is a natural polyaminosaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine and N-acetyl-D-glucosamine, having a very similar structure to that of cellulose. This polymer is increasingly used in biomedical and industrial fields. During the last decades, chitosan has become an important tool in gene therapy due to its biocompatibility and low cytotoxicity. However there are some disadvantages such as low solubility in aqueous media, and regarding drug delivery, it shows low transfection efficiency, and low specialty on targeted disease [1, 2].

As such form, chitosan is not present in nature, but it can be easily extracted from chitin through alkali deacetylation of this natural polymer. To „become" chitosan, chitin should be at least 60% deacetylated. The deacetylation of chitin can be performed in two ways: (1) by chemical hydrolysis, which happens under severe alkaline conditions, or (2) by enzymatic hydrolysis through particular enzymes such as chitin deacetylase [3, 4]. Chitosan has a rigid crystalline structure due to hydrogen bond which are formed inter- and intra-molecular because of the amine and hydroxyl groups [5].

Being the second most abundant polymer found in nature (after cellulose), chitin is found as a component part of many organisms, from fungi to crustaceans, to algae and insect cuticles [6, 7]. At industrial scale, however, the two main sources are represented by fungal mycelia and crustacean shells [8]. From these two, the mushroom source is considered more advantageous if considering the controlled production environment, this leading to a better reproducibility of the final product [9]. Another important aspect from which the fungal source is more appreciated is the allergenic point of view. The resulted chitosan is considered to be more suitable for biomedical applications [10].

As a natural polymer, the characterization of chitosan is very important because chitosan may be contaminated with organic and inorganic compounds. One significant drawback of chitosan is the poor solubility in aqueous solutions, except in acidic medium, and this makes the analyses slightly difficult to perform. The most used methods in characterizing chitosan are UV-spectroscopy, pH-potentiometric titration, IR-spectroscopy, NMR spectroscopy colloidal titration and also enzymatic degradation and viscosimetry and size exclusion chromatography [11, 12].

Chitosan solubility limits greatly its applications. One of the most important water soluble derivatives of chitosan are made by alkylation of amino group, or derivatization with charged functions of hydroxyl groups from the chitosan backbone. Compared to chitosan, these by-products have enhanced physicochemical and biological properties with great potential in applications such as tissue engineering and drug delivery. [13].

In the last years, there were many attempts to replace petrochemical products with renewable, biological components. Natural occurring polymers such as cellulose, chitin, starch, collagen, gelatin, and alginate [14] are abundant candidates that represent a sustainable source as they could reduce the consumption of fossil fuels, and thus having a positive impact over the environment.

Maybe the most challenging work is equipping these biomaterials with the same properties of the synthetic products, from a functional perspective. Chitosan is regarded like

a unique biopolymer, and because of its intrinsic properties it is most valuable than any petrochemical equivalent.

Chitosan is the only naturally occurring positively charged biopolymer [15] and also exhibits many important intrinsic features such as antibacterial along with antifungal activity [16], mucoadhesive, hemostatic and analgesic properties [17].

Because in biomedical fields are used so many by-products of chitosan, it is very important to study their biodegradability. Researchers showed that chitosan can be biodegraded into non-toxic residues [18] and it is believed that the rate of its degradation is highly dependent on the molecular mass and also its deacetylation degree. Furthermore, there are studies that showed that, at some extent, biocompatibility of chitosan based-materials can take place in physiological medium [19, 20]. These important features make chitosan an appropriate and outstanding candidate for biomedical applications.

CHITOSAN BASED PRODUCTS

Chitosan derivatives for gene therapy

Gene therapy is a biomedical field that attracts many scientists attention. This application uses genetic materials (DNA, RNA), as a pharmaceutical agent in order to treat numerous diseases. The main mechanisms underlying gene therapy are: delivering missing genes, replacing the defective ones and disabling undesired gene expression [21]. Thereby, gene therapy has the ability of treating various diseases and the interest regarding this application is constantly increasing. However, there are some disadvantages when using genetic material. First of all, nucleic acids can be rapidly degraded by nucleases, also they have larger size, high anionic charge, poor cellular uptake, and non-specificity [22].
Trying to overcome these shortcomings, researchers use vectors in gene therapy for safely delivering genetic materials.

Cationic polymers, like chitosan, are considered suitable carriers for delivery of non-viral genetic materials [23]. These polymers can condense with genetic materials due to electrostatic interactions and then form polyplexes in order to facilitate the cellular uptake [24]. Moreover, scientist observed that polyplexes escape the lysosome degradation by triggering an osmotic swelling effect, this being possible due to amino group of polyplexes which are quick in enabling the cell to absorb protons [25].

Chitosan has various bioactivities due to the primary amino groups present in this polymer main chain and for this reason chitosan is extensively used in many biomedical fields, such as drug delivery and gene therapy and also the industrial fields, such as water treatment, heavy metal flocculants and foods industry benefit from it [26].

Chitosan is a polysaccharide soluble in acidic solution, the optimum pH is about 6.5 [26]. Its solubility it is highly dependent on the degree of deacetylation (DD). If DD is around 40%, chitosan can be soluble to a pH value equal to 9, whereas if the DD of chitosan is approximately 80%, then this polymer it is soluble only at a maximum pH value of 6.5. There are some other factors that influence chitosan solubility, such as molecular weight (MW) and ionic strength of the solution [1].

Due to chemical properties described above, chitosan was extensively used as a gene

carrier for gene therapy. In pharmaceutical and medical applications, chitosan is used as a non-viral cationic polymer because of its biodegradability to normal body constituents, biocompatibility, bacteriostatic, fungistatic, hemostatic, non-toxic, anticancerogen, anticholesteremic properties. Being a cationic polymer, chitosan protects negatively charged genetic material against nuclease degradation.

Various studies presented that chitosan/DNA polyplexes have been able to transfect into various cell types: human embryonic kidney cells (HEK293), cervical cancer cells (HeLa cell) [25], primary chondrocytes [27], Chinese hamster ovary cells (CHO-K1) [28], fibroblast cells (NIH 3T3) [29], epithelioma papulosum cyprinid cells (EPC) [30].

Chitosan 3D-scaffolds for tissue engineering

When fabricating implantable scaffolds scientist should pay attention to body compatibility, scaffold mechanical properties, porosity, morphology, as well as healing and tissue replacement ability [31].

Very important data presented in published research concluded that tissue engineering scaffolds should not induce acute or chronic effects, should be biodegradable, because the new tissue should be able to replace the biopolymer used. Another important feature that scaffolds should possess is surface properties that promote cell attachment, differentiation and proliferation [32, 33]. Research in this direction are so valuable and chitosan could be a relevant candidate for 3D-scaffolds knowing that the preparation of such biomaterials could lead to substitution for damaged tissue and organs [34-36].

Chitosan hydrogels

Hydrogels are very interesting biomaterials because their high content of water it makes them compatible with the majority of living tissues. These gels are formed of a liquid phase, usually water (sometimes adjuvants) and a solid phase (less than 10% of the total volume of the gel) that ensures the consistency of the gel [10]. Moreover, the hydrogels must be soft and flexible simulating soft body tissues, so it can minimize the damage that is made during implantation [37]. Besides biomedical scaffolds for tissue replacements, hydrogels are also used as drug and growth factor delivery [38-41].

There were three main types of hydrogels developed, one of them being *physically associated chitosan hydrogels*. These physically associated hydrogels are formed by reversible interactions (hydrogen bonds, hydrophobic interactions or electrostatic interactions) between polymer chains. The structure of the gel is given by these interactions, fewer interactions give a softer gel and a higher number of interactions will result in a tighter and stiffer gel.

One remarkable property of chitosan is that it can form gels all by itself, not needing any additive.

This process is possible due to the neutralization of chitosan amino groups, which leads to the inhibition of the repulsion between chitosan chains. As such, the chitosan hydrogel formation occurs due to hydrogen bonds, hydrophobic interactions and chitosan crystallites

[40, 42-44].

Chitosan hydrogels can also be formed by mixing chitosan with other water-soluble non-ionic polymers such as PVA (polyvinyl alcohol) [45-48]. Thermo-sensitive chitosan hydrogels can be formed by mixing raw chitosan with polyol salts such as glycerol phosphate disodium salt [49-52]. More stable hydrogels can be formed with polyethylene glycol) [53, 54].

When forming gels, positively charged chitosan interacts with negatively charged molecules (sulfates, phosphates and citrates ions) [55, 56]. The swelling capacity of the obtained hydrogel is given by the number of D-glucosamine units vs. N-acetyl-D-glucosamine units, as well as by the concentration and size of the anionic species presented in the hydrogel.

Studies have described chitosan hydrogels in combination with larger negatively charged molecules such as proteins (albumin, gelatin, keratin, collagen and fibroin), anionic polysaccharides (alginate, pectin, hyaluronic acid, heparin, xanthan, dextran sulfate, chondroitin sulfate, fucoidan), glycosaminoglycans and carboxymethyl cellulose [51, 57-66].

Chitosan sponges

Chitosan sponges, obtained by freeze drying, are very useful as wound healing materials, because of their ability of soaking wound exudates, and in the meantime inducing tissue regeneration. In bone tissue engineering, chitosan sponges are used as a filling material [67]. Such examples of chitosan sponges are chitosan/tricalcium phosphate (TCP) [68] and chitosan/collagen sponges [67], chitosan–ZnO composites which have a good swelling ration and show hemostatic activity [69].

Chitosan films

Chitosan films are easily prepared by wet casting chitosan salt solutions followed by drying, either using oven or infrared (IR) drying [70, 71]. One example is Hem-Con bandage [72, 73] which is an engineered chitosan acetate derivative designed as a haemostatic dressing.

Chitosan porous nanofibers

Chitosan fibers can be produce in several ways, one of the first examples was reported in the early 1930, fibers were produce from acetic acid using dry and wet spinning [74-76]. In order to decrease the costs and improving fiber properties, chitosan was blended with other polymers such as sodium alginate [77], polyacrylic acid [78], sodium chondroitin sulfate, sodium heparin, sodium hyaluronat, cellulose [79].

In the recent years, the most used technique for preparations of nanofiber membranes is electrospinning (ESP). This is a versatile technique which produces polymer fibers from a few nanometers to microns in diameter [4].

BIODEGRADABILITY MECHANISM

When explaining chitosan biodegradability, it is important to know that chitosan, besides being a polymer bearing amino groups, it is also a polysaccharide, thus containing breakable glycosidic bonds. Chitosan seems to be degraded in vivo by unspecific enzymes, but mainly lysozyme was reported to have a such property [37, 80].

Biodegradation of chitosan leads to non-toxic oligosaccharides formation. It is possible that these oligosaccharides, with variable lengths, can be either incorporated in metabolic pathways or be excreted.

Zhang et al. observed that there is a link between degradation rate and molecular mass, deacetylation degree (DD) and the distribution of N-acetyl D-glucosamine residues [81]. The relation between chitosan biodegradability and DD is also dependent on crystallinity, chitosan being a semi-crystalline polymer. Crystallinity has its maximum at a DD equal to 0 (chitin form) or 100% (fully deacetylated chitosan), and it decreases at an intermediate DD. The biodegradation rate is increasing when crystallinity decreases, and this happens closely to 60% DD of chitosan.

Acetyl residues distributed along chitosan chain also affect the crystallinity of chitosan, and consequently, the biodegradation rate. It is appropriate to conclude that the smaller chitosan chains are more efficiently biodegraded that higher molecular mass chitosans [12, 82].

Although many tests have shown that chitosan is biocompatible and presents low toxicity [83, 84], FDA (Food and Drug Administration) only approved the use of chitosan as wound dressing [85].

Preparation method is crucial in determining biocompatibility of chitosan with physiological medium. One of the major concern related with biocompatibility it to avoid allergic reactions, that can be caused by residual proteins. The biocompatibility seems to be influenced by the density of amino groups on the polymeric chain, i.e. the biocompatibility increases with DD. Compared to chitin, chitosan proved to be more biocompatible *in vitro*. Apparently, with the increase in the number of positive charges, the interaction between chitosan and cells increases also, thus improving biocompatibility [86].

Being a natural biodegradable biopolymer, chitosan undergoes enzymatic degradation to non-toxic components.

In vivo, chitosan may be degraded by several enzymes, first of all by lysozyme which is a non-specific enzyme present in all mammalian tissues. Degradation products are non-toxic oligosaccharides which can be afterwards either excreted or incorporated to glycosaminoglycans and glycoproteins [87].

In vitro degradations of chitosan happens via oxidation, chemical, or enzymatic hydrolysis. These methods are commonly used for the preparation of low molecular chitosan under controlled conditions [88]. A crucial role in degradation rate is represented by molecular weight, deacetylation degree, polydispersity, purity level and moisture content.

The possible mechanism of degradation of chitosan usually begins with random splitting of β-1,4-glycosidic bonds (depolymerization) followed by hydrolysis of N-acetyl linkage (deacetylation). Consequently, molecular weight decreases and an increase in deacetylation degree is observed. Simultaneously, the cleavage of chitosan functional groups (amino, carbonyl, amido, and hydroxyl) may occurs, depending of the chemical and / or enzymatic

conditions. In addition, chitosan depolymerization may induce formation of free radicals which can lead to oxidation processes [89].

The polymer structure is altered by strong intermolecular interactions between formed fragments of chitosan, thus leading to the irreversible loss of its physicochemical properties.

Although numerous data have been published regarding chitosan application in biomedical fields, very few studies and review articles have investigated the long term stability of chitosan based products [90].

4. CONCLUSIONS

When using chitosan in applications for controlled drug delivery and tissue engineering it is required that, this polymer must be not only biocompatible but also biodegradable. Understanding of the biodegradation of chitosan is not only crucial but also necessary, because it involves performance and safety issues. Degradation rate of chitosan-based products depends on their applications. For example, when using chitosan-based product as a template in tissue-engineering applications, the degradation rate of the chitosan derivative should be relatively slow, because it should maintain its mechanical strength until tissue regeneration is almost complete. On the other side, if chitosan is used as a drug delivery carrier, then it should degrade relatively quickly but in a controlled manner in order to release continuously the drug to the target. The chemical structure also influences the biodegradation rate of chitosan, and also the MW, and the surrounding media to which it is applied. The degradation kinetics could affect the cell growth, tissue regeneration, and host response.

Biodegradation is even more important when chitosan derivatives are to be used in humans as drug delivery carriers or as tissue-engineering scaffolds. Biodegradation can be investigated using *in vitro* biodegradation methods, followed by *in vivo* models. The hydrolysis of chitosan and its derivatives, and thus biodegradation of the biomaterials in humans, is mainly catalyzed by lysozymes, chitosanases, chitinases, and chitin deacetylase enzymes. The MW and DD of chitosan are the dominant factors affecting the rate of biodegradation. High MW and DD it is believed to contribute to slow degradation, whereas low MW and DD enhances the biodegradation rate.

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REFERENCES

1. Choi, C., J.-P. Nam, and J.-W. Nah, *Application of chitosan and chitosan derivatives as biomaterials.* Journal of Industrial and Engineering Chemistry, 2016. **33**: p. 1-10.
2. Berezin, A.S. and Y.A. Skorik, *Chitosan-isoniazid conjugates: Synthesis, evaluation of tuberculostatic activity, biodegradability and toxicity.* Carbohydr Polym, 2015. **127**: p. 309-15.
3. Venkatesan, J. and S.K. Kim, *Chitosan composites for bone tissue engineering--an overview.* Mar Drugs, 2010. **8**(8): p. 2252-66.
4. Jayakumar, R., et al., *Biomedical applications of chitin and chitosan based nanomaterials—A short review.* Carbohydrate Polymers, 2010. **82**(2): p. 227-232.
5. Hejazi, R. and M. Amiji, *Chitosan-based gastrointestinal delivery systems.* Journal of Controlled Release, 2003. **89**(2): p. 151-165.
6. Aranaz, I., R. Harris, and A. Heras, *Chitosan amphiphilic derivatives. Chemistry and applications.* Current Organic Chemistry, 2010. **14**(3): p. 308-330.
7. Kumari, S. and P.K. Rath, *Extraction and Characterization of Chitin and Chitosan from (Labeo rohit) Fish Scales.* Procedia Materials Science, 2014. **6**: p. 482-489.
8. Kannan, M., et al., *Production and characterization of mushroom chitosan under solid-state fermentation conditions.* Adv Biol Res, 2010. **4**(1): p. 10-13.
9. Wu, T., et al., *Chitin and chitosan value-added products from mushroom waste.* Journal of agricultural and food chemistry, 2004. **52**(26): p. 7905-7910.
10. Riva, R., et al., *Chitosan and chitosan derivatives in drug delivery and tissue engineering*, in *Chitosan for biomaterials II*. 2011, Springer. p. 19-44.
11. Balázs, N. and P. Sipos, *Limitations of pH-potentiometric titration for the determination of the degree of deacetylation of chitosan.* Carbohydrate research, 2007. **342**(1): p. 124-130.
12. Rinaudo, M., *Chitin and chitosan: Properties and applications.* Progress in Polymer Science, 2006. **31**(7): p. 603-632.
13. Faizuloev, E., et al., *Water-soluble N-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride as a nucleic acids vector for cell transfection.* Carbohydr Polym, 2012. **89**(4): p. 1088-94.
14. Liu, X., et al., *Chitosan-Based Biomaterials for Tissue Repair and Regeneration*, in *Chitosan for Biomaterials II*, R. Jayakumar, M. Prabaharan, and R.A.A. Muzzarelli, Editors. 2011, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 81-127.
15. Pavinatto, F.J., L. Caseli, and O.N. Oliveira Jr, *Chitosan in nanostructured thin films.* Biomacromolecules, 2010. **11**(8): p. 1897-1908.
16. 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.
17. Yang, J., et al., *Effect of chitosan molecular weight and deacetylation degree on hemostasis.* Journal of Biomedical Materials Research Part B: Applied Biomaterials, 2008. **84**(1): p. 131-137.
18. Bagheri-Khoulenjani, S., S.M. Taghizadeh, and H. Mirzadeh, *An investigation on the short-term biodegradability of chitosan with various molecular weights and degrees of deacetylation.* Carbohydrate Polymers, 2009. **78**(4): p. 773-778.
19. VandeVord, P.J., et al., *Evaluation of the biocompatibility of a chitosan scaffold in mice.* Journal of Biomedical Materials Research Part A, 2002. **59**(3): p. 585-590.
20. Sashiwa, H. and S.-i. Aiba, *Chemically modified chitin and chitosan as biomaterials.* Progress in polymer science, 2004. **29**(9): p. 887-908.
21. Layek, B. and J. Singh, *Amino Acid Grafted Chitosan for High Performance Gene Delivery: Comparison of Amino Acid Hydrophobicity on Vector and Polyplex Characteristics.* Biomacromolecules, 2013. **14**(2): p. 485-494.

22. Jiang, H.-L., et al., *Chitosan-graft-spermine as a gene carrier in vitro and in vivo.* European Journal of Pharmaceutics and Biopharmaceutics, 2011. **77**(1): p. 36-42.
23. Liang, Y., et al., *Delivery of cationic polymer-siRNA nanoparticles for gene therapies in neural regeneration.* Biochemical and Biophysical Research Communications, 2012. **421**(4): p. 690-695.
24. Gao, X., et al., *Polyhydroxylalkyleneamines: A class of hydrophilic cationic polymer-based gene transfer agents.* Journal of Controlled Release, 2009. **137**(1): p. 38-45.
25. Kiang, T., et al., *The effect of the degree of chitosan deacetylation on the efficiency of gene transfection.* Biomaterials, 2004. **25**(22): p. 5293-5301.
26. Dong, C., W. Chen, and C. Liu, *Flocculation of algal cells by amphoteric chitosan-based flocculant.* Bioresource Technology, 2014. **170**: p. 239-247.
27. Zhao, X., et al., *Transfection of primary chondrocytes using chitosan-pEGFP nanoparticles.* Journal of Controlled Release, 2006. **112**(2): p. 223-228.
28. Weecharangsan, W., et al., *Evaluation of chitosan salts as non-viral gene vectors in CHO-K1 cells.* International Journal of Pharmaceutics, 2008. **348**(1): p. 161-168.
29. Niu, L., et al., *Expression of human insulin gene wrapped with chitosan nanoparticles in NIH3T3 cells and diabetic rats.* Acta Pharmacol Sin, 2008. **29**(11): p. 1342-1349.
30. Romøren, K., et al., *The influence of formulation variables on in vitro transfection efficiency and physicochemical properties of chitosan-based polyplexes.* International Journal of Pharmaceutics, 2003. **261**(1): p. 115-127.
31. Dutta, P., K. Rinki, and J. Dutta, *Chitosan: A promising biomaterial for tissue engineering scaffolds*, in *Chitosan for Biomaterials II*. 2011, Springer. p. 45-79.
32. elena OLTEANU, C. and D. ENESCU, *Chitosan involved Tissue Engineering and Regenerative Medicine.*
33. Zhu, X., et al., *Effect of argon-plasma treatment on proliferation of human-skin-derived fibroblast on chitosan membrane in vitro.* J Biomed Mater Res A, 2005. **73**(3): p. 264-74.
34. Jagur-Grodzinski, J., *Biomedical applications of polymers 2001-2002.* e-Polymers, 2003. **3**(1): p. 141-176c.
35. Kievit, F.M., et al., *Chitosan–alginate 3D scaffolds as a mimic of the glioma tumor microenvironment.* Biomaterials, 2010. **31**(22): p. 5903-5910.
36. Duarte, A.R.C., J.F. Mano, and R.L. Reis, *Novel 3D scaffolds of chitosan–PLLA blends for tissue engineering applications: Preparation and characterization.* The Journal of Supercritical Fluids, 2010. **54**(3): p. 282-289.
37. Dash, M., et al., *Chitosan—A versatile semi-synthetic polymer in biomedical applications.* Progress in polymer science, 2011. **36**(8): p. 981-1014.
38. Muzzarelli, R. and C. Muzzarelli, *Chitosan chemistry: relevance to the biomedical sciences*, in *Polysaccharides I*. 2005, Springer. p. 151-209.
39. Sayyar, S., et al., *Processable conducting graphene/chitosan hydrogels for tissue engineering.* Journal of Materials Chemistry B, 2015. **3**(3): p. 481-490.
40. Nie, J., et al., *Orientation in multi-layer chitosan hydrogel: morphology, mechanism, and design principle.* Scientific reports, 2015. **5**: p. 7635.
41. Gao, L., et al., *Effects of genipin cross-linking of chitosan hydrogels on cellular adhesion and viability.* Colloids and Surfaces B: Biointerfaces, 2014. **117**: p. 398-405.
42. Dhanasingh, A. and J. Groll, *Polysaccharide based covalently linked multi-membrane hydrogels.* Soft Matter, 2012. **8**(5): p. 1643-1647.
43. Duan, J., et al., *Versatile fabrication of arbitrarily shaped multi-membrane hydrogels suitable for biomedical applications.* Journal of Materials Chemistry B, 2013. **1**(4): p. 485-492.
44. Ladet, S., L. David, and A. Domard, *Multi-membrane hydrogels.* Nature, 2008. **452**(7183): p. 76-79.
45. Sung, J.H., et al., *Gel characterisation and in vivo evaluation of minocycline-loaded wound dressing with enhanced wound healing using polyvinyl alcohol and chitosan.* International journal of pharmaceutics, 2010. **392**(1): p. 232-240.

46. Berger, J., et al., *Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications.* European Journal of Pharmaceutics and Biopharmaceutics, 2004. **57**(1): p. 19-34.
47. Rafique, A., et al., *Chitosan functionalized poly (vinyl alcohol) for prospects biomedical and industrial applications: a review.* International journal of biological macromolecules, 2016. **87**: p. 141-154.
48. Garnica-Palafox, I. and F. Sanchez-Arevalo, *Influence of natural and synthetic crosslinking reagents on the structural and mechanical properties of chitosan-based hybrid hydrogels.* Carbohydrate polymers, 2016. **151**: p. 1073-1081.
49. Chenite, A., et al., *Novel injectable neutral solutions of chitosan form biodegradable gels in situ.* Biomaterials, 2000. **21**(21): p. 2155-2161.
50. Chenite, A., et al., *Rheological characterisation of thermogelling chitosan/glycerol-phosphate solutions.* Carbohydrate polymers, 2001. **46**(1): p. 39-47.
51. Cheng, Y.-H., et al., *Thermosensitive chitosan–gelatin–glycerol phosphate hydrogels as a cell carrier for nucleus pulposus regeneration: an in vitro study.* Tissue Engineering Part A, 2009. **16**(2): p. 695-703.
52. Kim, S., et al., *A chitosan/β-glycerophosphate thermo-sensitive gel for the delivery of ellagic acid for the treatment of brain cancer.* Biomaterials, 2010. **31**(14): p. 4157-4166.
53. Shantha, K. and D. Harding, *Preparation and in-vitro evaluation of poly [N-vinyl-2-pyrrolidone-polyethylene glycol diacrylate]-chitosan interpolymeric pH-responsive hydrogels for oral drug delivery.* International journal of pharmaceutics, 2000. **207**(1): p. 65-70.
54. Zhong, C., et al., *Synthesis, characterization and cytotoxicity of photo-crosslinked maleic chitosan–polyethylene glycol diacrylate hybrid hydrogels.* Acta biomaterialia, 2010. **6**(10): p. 3908-3918.
55. Shen, E.C., et al., Tetracycline release from tripolyphosphate–chitosan cross‐linked sponge: a preliminary in vitro study. Journal of periodontal research, 2008. 43(6): p. 642-648.
56. Shu, X. and K. Zhu, *Controlled drug release properties of ionically cross-linked chitosan beads: the influence of anion structure.* International Journal of Pharmaceutics, 2002. **233**(1): p. 217-225.
57. Jiang, T., et al., *Surface functionalization of titanium with chitosan/gelatin via electrophoretic deposition: characterization and cell behavior.* Biomacromolecules, 2010. **11**(5): p. 1254-1260.
58. Huang, X., et al., *Influence of radiation crosslinked carboxymethyl-chitosan/gelatin hydrogel on cutaneous wound healing.* Materials Science and Engineering: C, 2013. **33**(8): p. 4816-4824.
59. Nagahama, H., et al., *Preparation and characterization of novel chitosan/gelatin membranes using chitosan hydrogel.* Carbohydrate polymers, 2009. **76**(2): p. 255-260.
60. Bhattarai, N., J. Gunn, and M. Zhang, *Chitosan-based hydrogels for controlled, localized drug delivery.* Advanced drug delivery reviews, 2010. **62**(1): p. 83-99.
61. 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.
62. Cui, F., et al., *Development of chitosan-collagen hydrogel incorporated with lysostaphin (CCHL) burn dressing with anti-methicillin-resistant Staphylococcus aureus and promotion wound healing properties.* Drug delivery, 2011. **18**(3): p. 173-180.
63. Belščak-Cvitanović, A., et al., *Improving the controlled delivery formulations of caffeine in alginate hydrogel beads combined with pectin, carrageenan, chitosan and psyllium.* Food chemistry, 2015. **167**: p. 378-386.
64. Martins, A.F., et al., *Bactericidal activity of hydrogel beads based on N, N, N-trimethyl chitosan/alginate complexes loaded with silver nanoparticles.* Chinese Chemical Letters, 2015. **26**(9): p. 1129-1132.

65. Xu, J., et al., *Genipin-crosslinked catechol-chitosan mucoadhesive hydrogels for buccal drug delivery.* Biomaterials, 2015. **37**: p. 395-404.
66. Ahmadi, F., et al., *Chitosan based hydrogels: characteristics and pharmaceutical applications.* Research in pharmaceutical sciences, 2015. **10**(1): p. 1.
67. Costa-Pinto, A.R., R.L. Reis, and N.M. Neves, *Scaffolds based bone tissue engineering: the role of chitosan.* Tissue Engineering Part B: Reviews, 2011. **17**(5): p. 331-347.
68. Seol, Y.-J., et al., *Chitosan sponges as tissue engineering scaffolds for bone formation.* Biotechnology letters, 2004. **26**(13): p. 1037-1041.
69. Jayakumar, R., et al., *Biomaterials based on chitin and chitosan in wound dressing applications.* Biotechnology advances, 2011. **29**(3): p. 322-337.
70. Youssef, A.M., et al., *Mechanical and antibacterial properties of novel high performance chitosan/nanocomposite films.* International journal of biological macromolecules, 2015. **76**: p. 25-32.
71. Ahmed, M.G., et al., *Formulation and in-vitro evaluation of Chitosan films containing tetracycline for the treatment of periodontitis.* Asian Journal of Pharmaceutics (AJP): Free full text articles from Asian J Pharm, 2014. **3**(2).
72. Moharana, A., *Polyelectrolyte Complex Composed of Chitosan and Sodium Alginate for Wound Healing*. 2015.
73. Ebenezer, V. and R. Balakrishnan, *Impact of Hemostatic Agents in Oral Surgery.* Biomedical and Pharmacology Journal, 2015. **7**(1): p. 215-219.
74. 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.
75. *Process for the preparation of films and filaments and products thereof.* 1936, Google Patents.
76. East, G.C. and Y. Qin, *Wet spinning of chitosan and the acetylation of chitosan fibers.* Journal of applied polymer science, 1993. **50**(10): p. 1773-1779.
77. Sibaja, B., et al., *Preparation of alginate–chitosan fibers with potential biomedical applications.* Carbohydrate polymers, 2015. **134**: p. 598-608.
78. Ohkawa, K., et al., *Preparing chitosan-poly (acrylic acid) composite fibers by self-assembly at an aqueous solution interface.* Textile research journal, 2002. **72**(2): p. 120-124.
79. Hirano, S., et al., *Wet spun chitosan–collagen fibers, their chemical N-modifications, and blood compatibility.* Biomaterials, 2000. **21**(10): p. 997-1003.
80. Kean, T. and M. Thanou, *Chitin and chitosan: sources, production and medical applications.* Renewable resources for functional polymers and biomaterials, 2011: p. 292-318.
81. Zhang, H. and S.H. Neau, *In vitro degradation of chitosan by a commercial enzyme preparation: effect of molecular weight and degree of deacetylation.* Biomaterials, 2001. **22**(12): p. 1653-1658.
82. Croisier, F. and C. Jérôme, *Chitosan-based biomaterials for tissue engineering.* European Polymer Journal, 2013. **49**(4): p. 780-792.
83. Thanou, M., J. Verhoef, and H. Junginger, *Oral drug absorption enhancement by chitosan and its derivatives.* Advanced drug delivery reviews, 2001. **52**(2): p. 117-126.
84. Aranaz, I., et al., *Functional characterization of chitin and chitosan.* Current chemical biology, 2009. **3**(2): p. 203-230.
85. Wedmore, I., et al., *A special report on the chitosan-based hemostatic dressing: experience in current combat operations.* J Trauma, 2006. **60**(3): p. 655-8.
86. Chatelet, C., O. Damour, and A. Domard, *Influence of the degree of acetylation on some biological properties of chitosan films.* Biomaterials, 2001. **22**(3): p. 261-268.
87. Kurita, K., et al., *Enzymatic degradation of β-chitin: susceptibility and the influence of deacetylation.* Carbohydrate Polymers, 2000. **42**(1): p. 19-21.
88. Ma, Z., et al., *Oxidative degradation of chitosan to the low molecular water-soluble chitosan over peroxotungstate as chemical scissors.* PLoS One, 2014. **9**(6): p. e100743.

89. Mucha, M. and A. Pawlak, *Complex study on chitosan degradability*. POLIMERY-WARSAW-, 2002. **47**(7/8): p. 509-516.
90. Szymańska, E. and K. Winnicka, *Stability of Chitosan—A Challenge for Pharmaceutical and Biomedical Applications*. Marine Drugs, 2015. **13**(4): p. 1819-1846.