Biomimetic scaffolds based on chitosan in bone regeneration. A review.

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Abstract

The article focuses on a polysaccharide of natural origin – chitosan and its application in tissue engineering. The preparation process and physicochemical properties of the saccharide are described. The degradation of chitosan and the properties influencing the process both outside and in living organism were examined. Four applications in bone tissue engineering can be distinguished: preparation of cell scaffolds exclusively from chitosan, from a chitosan composite or from a chitosan polyelectrolyte complex. The fourth way is to modify the surface of scaffolds made of other materials by covering them with a layer of chitosan. At the end of the article, the processes taking place after placing the implant inside the body are described, how the structure of chitosan affects the behaviour of bone cells in the adhesion process and life processes.

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ABSTRACT:

The article focuses on a polysaccharide of natural origin – chitosan and its application in tissue engineering. The preparation process and physicochemical properties of the saccharide are described. The degradation of chitosan and the properties influencing the process both outside and in living organism were examined. Four applications in bone tissue engineering can be distinguished: preparation of cell scaffolds exclusively from chitosan, from a chitosan composite or from a chitosan polyelectrolyte complex. The fourth way is to modify the surface of scaffolds made of other materials by covering them with a layer of chitosan. At the end of the article, the processes taking place after placing the implant inside the body are described, how the structure of chitosan affects the behaviour of bone cells in the adhesion process and life processes.

INTRODUCTION

Bone lesions are common injuries. In some cases, with a sufficiently small size of the defect, the bone is able to regenerate itself.[1] In optimal conditions, healing can take place without scarring, i.e. in such a way that the regenerated tissue is indistinguishable from the state before the damage.[2]There are also injuries that cannot regenerate spontaneously, despite surgical intervention and stabilisation, they require special treatment. Such injuries are called critical defects. It is assumed that the size of such lesions is approx. 1–2 cm and more than 50% of the bone volume is affected.[3] Regeneration of such defects is complicated, difficult to control, until now it has involved autologous bone transplant surgery.[4] This technique is relatively good

due to its histocompatibility and non-immunogenicity. It also has its disadvantages, such as the formation of damage at the site of extraction of the implant tissue, risk of secondary damage, scarring, distortion, and others.[5,6] Tissue engineering is also involved in the treatment of such critical lesions and is an alternative to conventional transplants. It is possible to use scaffolds made from biomaterials, which will provide a place for attachment for new bone cells and form the basis for the reconstruction process.[7,8]

Biomaterials are the basic materials used in tissue engineering. It is a group of compounds that can come into direct contact with tissues and are well tolerated by the body. Biomaterials must be biocompatible, effective and sterilisable.[9,10] There are three generations of biomaterials, chronologically these are inert materials, bioactive materials and biomimetic materials. The inert materials replace the damaged organ or its fragment without interacting with the body. Bioactive materials combine with tissue through biochemical reactions and can replace the damaged element. Biomimetic materials are designed to stimulate the growth and proliferation of cells on the surface of the material. They are characterised by both bioactivity and bioresorbability. This group includes biodegradable scaffolds, which are a place for cell attachment, later tissue development and decay over time.[11,12] The aim of the literature review was to study the popular polysaccharide of natural origin – chitosan, its properties, behaviour in contact with body cells and applications in bone tissue engineering.

Chitosan

Chitosan (CS) is a derivative of the natural polysaccharide – chitin (Figure 1). CS is obtained by partial deacetylation of chitin in which at least half of the acetyl groups are removed. Chitosan is a linear cationic polymer, a copolymer composed of*N*– acetyl–*D*– glucosamine and *D*– glucosamine units linked by β-1,4-glycosidic bonds.

Preparation and chemical structure

The source of chitosan is chitin – the second most common polysaccharide in nature.[13] CI is one of the main building blocks of arthropod skeletons, some molluscs and fungal cell walls.[14,15] Industrial chitin is most often obtained from food industry waste, crustaceans or sea molluscs (shrimps, lobsters, crabs, squid, mussels),[16–19] it may also be of fungal origin.[20]

Obtaining chitin

Due to its low solubility in popular solvents, chitin is obtained as a result of a multi-stage extraction process (chemical purification). The steps in this process are demineralisation , deproteinisation, and discolouration may also be performed (Figure 2).

The dried shells of crustaceans are demineralised by acid to remove minerals, especially calcium (hence in some sources this stage is called decalcination). The most common is an aqueous HCl solution (up to 10% concentration), but also HNO_3, H_2SO_4, HCOOH or a more environmentally friendly acetic acid solution are commonly used.[21,22]The insoluble residue is centrifuged and then repeatedly washed with water until the pH is neutral. In the deproteinisation step, the residues are treated with alkalines in order to remove proteins from them. Most often, solutions of monohydroxy bases are used, e.g. NaOH, KOH, but also salt solutions such as Na_2CO_3, $NaHCO_3$, K_2CO_3, $NaHSO_3$.[23] The fractions are separated again, washed with water until neutral pH and dried. Thus obtained chitin has a pinkish colour.[13] Chitin discolouration takes place under the influence of an oxidant, e.g. $KMnO_4$, H_2O_2, then the precipitate is washed with a solution of oxalic acid. In this way, colourless, pure chitin is obtained.[13,22] It is also possible to purify chitin biologically with the use of enzymes – proteases[24–26] or microbes in the fermentation process.[27]

Preparation of chitosan

The purified chitin is subjected to a deacetylation process (Figure 2). To this end, the CI is treated with a concentrated base, most often NaOH,[21] and then washed with water and dried. The chitosan thus obtained is in the form of a white powder.[13]

In the deacetylation process random acetyl groups are removed in favour of the amino group, chitosan is a copolymer of*N*– acetyl–*D*– glucosamine and D–glucosamine units. The presence of the latter groups depends on the degree of deacetylation (DD) that is used to characterise the CS. The more deacetylated the CS is, the fewer *N*– acetyl–*D*– glucosamine groups. The value of the DD of chitosan is in the range of 50–95%.[17,28]

Physicochemical properties

In its structure, chitosan contains functional groups: hydroxyl, amine and acetyl. They have a significant impact on the chemical properties of the polymer and the nature of interactions. The presence of hydrophobic acetyl groups and hydrophilic amine and hydroxyl groups ensures the amphiphilic nature of the polymer.[13] The amine groups that are responsible for the cationic nature of the polymer result in a high surface tension of aqueous solutions of this polysaccharide.[29]

CS is insoluble in water, in popular organic solvents (including methanol, ethanol, isopropanol, toluene), however, it dissolves in dilute aqueous acid solutions, e.g. in hydrochloric acid, acetic acid, formic acid, citric acid.[30–32] This property is due to the presence of free amino groups that can protonate at a pH below 6.[33] The amine groups become positively changed, breaking the hydrogen bonds and allowing the polymer to dissolve. Above this pH value, the $–NH_3^+$ groups deprotonate, lose their charge, and the polymer solubility decreases.[16,34] The results of the solubility tests are presented in Table 1.[30]

The solubility of chitosan also depends on the degree of deacetylation (DD) and the molecular weight of the polysaccharide. The more amino groups there are, i.e. the higher the deacetylation degree, the better the chitosan solubility.[23] The higher the molecular weight of the polymer, the more internal hydrogen bonds there are and the more difficult it is to dissolve.[17] CS forms water-soluble salts, including formates, acetates, hydrochlorides, citrates.[35] To characterise chitosan, its viscosity is often used, which depends on various parameters such as concentration of chitosan in the solution, temperature, degree of deacetylation.[36]

The cationic nature of chitosan distinguishes it from other natural polysaccharides. Due to its positive charge, chitosan has the ability to form complexes with negatively charged compounds, e.g. polylactide[37], poly(glutamic acid)[38], DNA[39,40], collagen[41], alginates[42–44], hyaluronic acid[45–47], pectin[48,49]and many others.

The structure of chitosan, which is based on *D*– glucosamine and*N*– acetyl–*D*– glucosamine units, is similar to the structure of naturally occurring glycosaminoglycans (GAG). GAG can build the extracellular matrix and perform various functions in organisms, e.g. they affect adhesion, growth factors and cell receptors, water retention (influencing the resistance of cells to compression). It can be expected that, thanks to its GAG-like structure, chitosan can also interact with animal cells.[7,34]

Chitosan is readily used in tissue engineering because it is characterised by features desired in biomaterials: biocompatibility, non-toxicity, the ability to biodegrade into non-toxic compounds and bioactivity.[50–52] It also demonstrates osteocompatibility (the presence of chitosan does not adversely affect bone regeneration) and osteoconduction (the ability to connect with bone tissue and stimulate its growth).[13] CS also has mucoadhesive properties, i.e. it promotes the adhesion of animal mucus cells to the biomaterial. This property is due to the difference in charges between positive chitosan and negative mucus, between which electrostatic interactions are generated.[53,54] CS is also credited with the ability to inhibit the growth of neoplastic cells[55,56] and promote wound healing.[57–60] Chitosan also has antibacterial and antifungal properties (against Gram-positive [61] and Gram-negative bacteria[62]). These properties are probably due to the interaction between the positively charged polymer and negatively charged lipopolysaccharides or proteins in microbial membranes.[59] Interactions with the surface of the bacterial cell reduce the permeability. The antibacterial properties of chitosan may also result from the interaction with the DNA of the bacterial cell, which results in inhibition of RNA synthesis.[50,59,63]

Chitosan degradation

Chitosan is a natural polymer which decomposes under the influence of both chemical and physical factors and enzymes. Chemical factors include depolymerisation under the influence of strong acids, e.g. HCl, HNO_2, H_3PO_4, CH_3COOH,[64–66] strong oxidants[67] or free radicals.[68] The products of such reactions are

chitosan oligosaccharides with various, low molecular weights.[69] Chemical degradation is a fast process, but expensive, poorly controlled (random cutting of the polymer chain[70]) and highly polluting towards the environment due to the chemical compounds used.[55,65,71]

Physical methods of CS depolymerisation include, among others, γ radiation, ultrasound. Contrary to chemical depolymerisation, mechanical cuts occur more frequently inside the polymer,[72] and the obtained oligosaccharides are characterised by a narrower size distribution.[69] Gamma radiation can be used to sterilise chitosan products. It was examined that under the influence of gamma radiation the CS structure changes, there is a decrease in molecular weight already at 15 kGy, which increases with radiation dosage.[73] Such changes may affect the properties of the polymer and, consequently, the chitosan-containing medical device. The use of ultrasound is a popular solution due to its low environmental impact. Degradation of chitosan by ultrasound is based on mechanical phenomena and depends on wave intensity, temperature, polymer concentration, ionic forces.[74] There are also studies on CS depolymerisation using the cavitation process.[69,75,76]

The use of enzymes for the depolymerisation of CS is associated with carrying out the reaction in mild conditions and fairly good control of the process. It is also an environmentally friendly method.[64,65] The enzyme specific chitosanase can be used in the hydrolysis of β-1,4-glycosidic bonds, as well as non-specific chitinases[77], lysozym[78]or cellulases.[71] The reduction of the CS molecular weight under the influence of the protease enzyme changed the structure of the oligomer, but did not affect the deacetylation degree.[79] Proteases can also be used for the production of monomers (*N*– acetyl–*D*– glucosamine units).[80] The use of lipase, the enzyme present in the human body, in CS depolymerisation leads to products with different molecular sizes in the 30 – 50 kDa range, without major structural changes to the polymer.[81]

Metabolism in living organisms

A biodegradable scaffold used in bone regeneration should degrade in the body into known, non-toxic products that can be excreted from the body or incorporated into the body's natural metabolic cycles. The time of scaffold degradation in the body depends on the place of implanting and the patient (their age, chronic diseases), but on average in bone regeneration it should take from 3 to 9 months.[82]Hydrolysis of glycosidic bonds in chitosan leads to the production of non-toxic glucosamine oligosaccharides, which can be rapidly cleared from the body in urine.[83,84] Chitosan implants do not elicit a long-lasting inflammatory or immune response.[52] The inflammation observed after the introduction of chitosan scaffolds depends on the degree of deacetylation of the polysaccharide. Chitosan with a lower DD induced a stronger leukocyte response.[85] The rate of degradation of chitosan implemented in the form of microspheres, hydrogels or 3D scaffolds varies. The onset of degradation of chitosan in the form of microspheres was observed 4 weeks after introduction into the body of rats, and after 12 weeks the microspheres disintegrated into small fragments.[86] For chitosan hydrogel, strong degradation was observed after 4 weeks.[87] In turn, the 3D porous scaffold made of chitosan after 30 days *in vivo* was completely biodegraded.[88] Hydrolysis of bonds in chitosan takes place in the human body under the influence of a non-specific enzyme lysozyme.[89] It has been noted that due to the cationic nature of chitosan, it can interact with blood cells. In the presence of chitosan, blood tends to coagulate and form clots on the surface of chitosan.[90] After oral administration of a polysaccharide in the organisms of rabbits, a specific response was observed, i.e. an increase in lysozyme secretion by the organism, which may prevent thrombosis.[91] *In vivo* degradation studies of chitosan-poly-L-lactide composites in rats showed that the polysaccharide did not cause an unexpected, negative response, no blood clots were detected after 1 week.[92]

Cell scaffolding

Tissue engineering is looking for solutions that could replace the need for autographic or allographic transplants. One of the proposed solutions is the use of scaffolds that mimic the basic roles of tissues and enable their regeneration.[93,94]

Such scaffolding is usually a three-dimensional, porous structure made of biomaterial. The main task of scaffolding is to support the regenerating tissue by providing a rack on the surface of which cells can settle.[8]

Scaffolds should imitate the tissue for which they are used to regenerate, its structure and properties.[95] The implant must not cause a negative immune response, it should be metabolised by the body, easy to shape and sterilise, and it should be durable so that it can be stored in *ex vivo* conditions.[96]

Scaffolds made of chitosan alone

Due to the properties of chitosan, such as non-toxicity, biocompatibility, affinity to animal mucus and biodegradability, it is a polymer very readily used in tissue engineering.[4] It supports cell adhesion and proliferation, and thus tissue regeneration. It allows you to easily model the scaffolding to a specific shape and create porous structures with open pores. These features make chitosan a potential candidate for scaffolding material.[97]

The temperatures used during the production of 3D scaffolding significantly affect, among others, the pore size or mechanical properties related to the compressive modulus.[98]Chitosan with a higher degree of deacetylation was characterised by higher mechanical resistance, slower degradation time and lower absorbability of the scaffold.[99] High DD (DD 88% and 95% tested) supports cell proliferation on the surface of the chitosan scaffold.[99] The porosity of the scaffold has a significant influence on its mechanical properties. The more porous the structure, the worse the mechanical properties. The decrease in the porosity from 94.1% to 82.5% was related to an increase in Young's modulus to 5.2 kPa and 520 kPa, respectively, while the pore size decreased. Cell adhesion was possible on both types of scaffolds, but better conditions were provided by scaffolds with lower porosity, which was associated with an increase in fibre density.[100]

Due to the properties of chitosan, it is a readily used biomaterial. In vitro and in vivo studies are conducted on the use of chitosan scaffolds in the treatment of damage to various tissues. The interactions and influence of CS on cells are investigated, among others, spinal cord[52], bones, cartilage[101], tendons[102], skin[103].

The use of chitosan in the treatment of the spinal cord has been studied. The implants were inserted into the meninges or directly into the spinal cord for 6 or 12 months, respectively. The host cell response (rat) was low, which indicates the inert nature of chitosan and the possibility of using the material in long-term therapies.[52] In search of chitosan with the best properties for the osteochondral treatment, porous chitosan scaffolds were prepared. *In vivo* studies indicate that the cellular response depended on the properties of the chitosan used, and chitosan with a DD of 83%, a relatively low molecular weight of 11.49 KDa and a high content of calcium, remaining due to the lack of demineralisation during the preparation, turned out to be the best for applications. After implant placement, its degradation and shaping of the subchondral bone were observed.[101]

Active layers can be applied to the scaffolds to improve the interaction at the biomaterial-tissue interface.[104] The surface of the chitosan scaffolds was mineralised to obtain apatite layers imitating bone. After modification, the scaffolds are characterised by greater rigidity and smaller pore sizes, yet sufficiently large to allow unhindered migration of bone cells.[105] The conducted*in vitro* studies indicate that the scaffold after apatite modification supports the adhesion and proliferation of cells to a greater extent than the unmodified version. Mineralised scaffolds are better suited for use in bone tissue engineering.[106,107]

The cultivation of chondrocyte cells is aided by the use of porous chitosan microspheres (porosity ˜90%, pore size 5 – 60 μm) with diameters of 180 – 280 μm[108] and CS fibres with diameters between 4 and 22 μm. The fibres maintained the viability of chondrocytes (over 85%) and supported the formation of the extracellular matrix (ECM). However, it was concluded that reducing the size and the use of nanofibers could improve interactions with chondrocytes.[109]

Chitosan as a scaffold surface modifier

Chitosan is often used as a coating material for implants or scaffolds. Such surface modification significantly improves the surface properties of an implant – its bioactivity, biocompatibility, corrosion resistance, as well as, properties supporting bone regeneration, such as osteoconductivity.[110] Chitosan layers are stable, and depending on the concentration of chitosan, its molecular weight, the degree of deacetylation and application technique, they degrade at different rates, even allowing for long-term use.[111,112]

Surface modification significantly influences the mechanical properties of the scaffolding. Three-dimensional, porous scaffolds made of polycaprolactam (PCL) were covered with layers of chitosan at various concentrations, from 1% to 3%. As the concentration of chitosan in the layer increases, the compression strength and the compression modulus factor increase, while scaffolding crystallinity decreases. The increase in the first two is likely related to the increase in the amount of amine groups that support PCL and improve the strength of the scaffold. The decrease in crystallinity is a result of the formation of hydrogen bonds between PCL and chitosan. Among the examined, the best osteoblast differentiation was provided by the PCL scaffold covered with a 2.5% chitosan layer.[113]

It is commonly known that the hydrophilicity/hydrophobicity of the scaffold affects cell adhesion and proliferation. Polylactide (PLA) is a polymer readily used in tissue engineering due to its biocompatibility and bioresorbability. It is however, a hydrophobic polymer that does not promote cell adhesion. In order to increase the hydrophilicity of the polymer, the PLA surface can be modified by applying a layer of chitosan.[114] Modification of poly-*L*- lactide surface with chitosan also improves mechanical properties of the scaffold, as well as improves adhesion, differentiation, activity, and morphology of chondrocyte cells[115] and mouse bone marrow stromal cells (mBMSCs) compared to the unmodified structure, which accelerates the bone regeneration process.[51,116]The introduction of the PLLA scaffold with a CS layer into skull defects of rats and the examination of bone changes after 12 weeks showed that the modified scaffolds support greater growth of bone tissue with higher density compared to the unmodified ones. This confirms the suspicions that chitosan-coated polylactide scaffolds are sustainable for bone regeneration,[116] and modifying the scaffold surface with chitosan significantly improves osteoblast adhesion and proliferation.[117]

Chitosan surfaces exhibit antimicrobial properties, highly desirable in biomedical applications. The antibacterial nature of CS was tested on Gram-negative strains of *E. coli* [118,119] and Gram positive *Staphylococcus aureus* .[120] For both groups, the use of chitosan layers reduced bacterial settling.

In addition to applying layers of chitosan itself, layers of mixtures of chitosan and other compounds are also used to improve certain properties. The use of a chitosan layer with ZnO particles on a titanium implant increases the compatibility of such an implant and its corrosion resistance, which results from the closure of pores on the implant surface. The introduction of the oxide improves the antibacterial properties of the layer compared to the layer made of chitosan itself.[119] Antibiotics can also be incorporated into the chitosan layer. The compounds bind through weak intermolecular interactions, which allows easy release of the antibiotic and, consequently, the fight against bacteria.[121]

Complexes with other natural polymers

Due to the cationic nature of the polymer, CS has the ability to spontaneously form stable polyelectrolyte complexes (PEC) with negatively charged structures such as natural polymers. In solution, permanent electrostatic interactions are formed between the cationic amino group of chitosan and the negatively charged group of the polyanion. Complexes are formed in stages, first the primary complex is formed, with chains of polyions randomly arranged in space. Then the chains arrange themselves in orderly structures, which is associated with the formation of hydrogen bonds, van der Waalss forces and others. Concurrently, aggregation into various structures may take place. The wide range of properties, as well as the non-toxicity, biodegradability and biocompatibility of PEC ensure a wide application of the complexes, especially in tissue engineering.[122,123] Examples of PEC are shown in Table 2. PEC formation can be investigated, for example, by FTIR (N–H, C=O bond shift study), DSC differential scanning calorimetry (melting point shift) or by examining changes in zeta potential.

The formation of complexes is influenced by the concentration of polymers, the mass ratio of polyelectrolytes, the order of polymer addition, the degree of CS deacetylation, the pH value of the solution, presence of other ions and molecules in the solution. As the concentration of polymers in the solution increases, the size of the particles formed increases. In turn, the mass ratio of the polymers used affects the size, charge and solubility of resulting complexes.[124,125] The molecular weight of chitosan and the degree of deacetylation affect the size of the complex. The larger the polymer, the thinner the packing of the compound and the larger the

size of the PEC.[39] Other molecules or ions than the polymers forming the complex may be present in the solution. They affect the ease of formation of complexes, their stability and properties. An example of such a compound is sodium lauryl sulphate surfactant, the use of which increases the surface roughness and the thickness of the chitosan/alginate (CS/Alg).[125,126]

The size of the nanoparticles of the chitosan CS/Pc with pectin complex varied depending on the order of addition of polymers. At low polymer concentrations, larger particles were formed when chitosan was added first, the inverse was true at high concentrations. The size of the complexes ranged from 460 nm (low polymer concentration) to 1110 nm (high polymer concentration). The zeta potential depends on the mass ratio of the polymers used, for complexes with different mass ratios it was in the range between 40 and 60 mV, and the advantage of chitosan in the complex increases the value of the potential, i.e. the stability of the complex. CS/Pc nanocomplexes are characterised by low stability compared to other polyelectrolyte complexes, they lose their stability after 14 days, and they degrade after 30 days. The stability is independent of particle size, but is dependent on the pH of the solution (stable at pH 3.5 – 6.0).[124]

The introduction of a polyanion into a chitosan scaffold has many advantages. The electrostatic interaction between the polymers increases the stability of the scaffolds obtained.[127]Modification with alginate improves the mechanical properties of the scaffold, the compressive modulus and yield strength after the modification are, respectively, 8.16 MPa and 0.46 MPa, for the chitosan only scaffold these values are about three times lower. Also, the preparation of the scaffold from the complex is simpler, since the environment is not necessarily acidic, it can just as well be alkaline or neutral. The chitosan/alginate complex is non-toxic, has anti-inflammatory effects,[128] in many cases supports adhesion, as well as osteoblast proliferation and angiogenesis.[129] However, some sources report the CS/Alg surface as unfavourable for cell adhesion.[43]

Composites with chitosan

Often, implants made of purely natural or synthetic polymers do not have the mechanical properties desired in tissue engineering, for instance, they are not able to carry the required loads.[13] To change the properties of the scaffold, a new compound can be introduced into the starting composition to obtain a composite. Examples of complexes are presented in Table 3. The use of the composite influences morphology, mechanical properties, porosity, swelling of the scaffolds as well as biomineralisation and degradation processes. Chitosan is a modifier commonly used in composites, a building material popular in the animal world, composites of which support bone regeneration processes such as adhesion and proliferation of bone cells, osteocalcin secretion or biomineralisation.[138–140] On the other hand, nanoparticles are readily introduced into chitosan scaffolds, thanks to which biomineralisation takes place more efficiently and the degradation rate decreases.

The morphology of the scaffolds significantly depends on the concentrations of the polymers used. For the poly-L-lactide + chitosan (PLLA+CS) scaffold, the higher the concentration of chitosan in the starting solution, the more chitosan was deposited on the pore surface, making the pores surface more jagged compared to polylactide systems alone, which is desirable due to an increase in the surface of the implant.[139]

The use of the PLLA+CS composite significantly affects the rate of scaffold degradation in comparison to the polylactide scaffold. This is explained by the fact that when PLLA hydrolyses into lactic acid, it lowers the pH of the environment, which causes the dissolution of the CS. In this way, along with the depletion of chitosan, the pores of the scaffold increase, which increases its surface contact with water. Also, the hydrophilic properties of chitosan accelerate the diffusion of water into the interior, making hydrolysis of the hydrophobic polylactide faster.[92]

The use of composites has a significant influence on mechanical properties. In order to strengthen the macrospheres made of chitosan, montmorillonite (MMT) and/or hydroxyapatite (HAP) were introduced into the system. Composite scaffoldings were characterised by much greater compressive strength. The introduction of MMT and HAP also reduced the swelling of scaffolds, which is probably related to the hydrophobicity of the additives and the binding of hydrophilic amino and hydroxyl CS groups by hydroxyapatite. Interactions with bone cells and cell proliferation supported all systems.[141]

The chitosan/collagen polyelectrolyte complex (CS/Coll) was enriched with MMT and modified organomontmorillonite (OMMT). The scaffolds made of PEC had weakly connected large pores (about 1 mm). The introduction of MMT and OMMT made it possible to obtain a structure with well connected pores of smaller diameters (200 – 400 μm for CS/Coll+MMT and 50 – 200 μm for CS/Coll+OMMT), which are better suited for seeding cells. Both composites were characterised by lower swelling ratio, degradation rates and greater compressive strength, and they supported biomineralisation better than the CS/Coll complex.[142]

The introduction of nanomaterials into scaffolds made of chitosan or its complexes affects the properties of the structures. The use of a chitosan-chitin composite with the addition of ZrO_2nanoparticles (CS+CI+nZrO_2) enables the production of non-toxic scaffolds with a pore size of 150 – 200 μm (larger in the CS+CI system) and improved bioactivity.[143] Porous scaffolds made of a combination of chitosan, gelatine and hydroxyapatite (Cs+Ge+nHAP composite) with an average pore size of 100 – 180 μm support the adhesion and development of bone cells. Hydroxyapatite occurs naturally in human bones, while gelatine and chitosan mimic the extracellular matrix (ECM) well. The introduction of nHAP significantly affects the properties of the system, increases the susceptibility to mineralisation of the scaffold, and as a result, significantly improves cytocompatibility, reduces swelling and the rate of degradation. Composite scaffolds are well suited for bone tissue engineering.[144] In the same way, the properties of the Cs+Ge scaffolds change after the introduction of SiO_2nanoparticles (Cs+Ge+nSiO_2composite).[145]

Cellular response to chitosan materials

After a scaffold is implanted, the body reacts, which ultimately leads to tissue reconstruction. The damage is immediately recognised and triggers appropriate interactions with blood, clot formation, cellular response, and eventually tissue reconstruction.[157] A thin layer of water forms on the scaffold surface, and proteins adhere to it. The bone cells then recognise these proteins and settle on the implant. Cell growth and integration enables tissue to regenerate on the scaffold surface.[158,159] Depending on the surface chemistry and implant topography, cells may interact with the material with different intensity.

Cell adhesion

The term "adhesion" describes two phenomena that occur during the settlement of scaffolding. It concerns the short-term generation of interactions between the cell membrane and the material (ionic forces, van der Walls interactions) and long-term cell attachment.[159] Biomaterials may affect cell adhesion, be osteoconductive or osteoinductive. Osteoconduction is the ability of a material to connect to bone tissue, provide adequate support for cells and influence the direction of the regeneration process. The osteoconductive material also positively influences the processes of cell proliferation and ECM formation. Osteoinduction involves triggering a series of processes and reactions that lead to bone regeneration through the use of biomolecular signalling devices. One such process is, for example, cell differentiation.[160–162]

Implants are recognised by the body as foreign bodies and trigger an immune response. The body's response mechanism after implantation (Figure 3) begins with the formation of a thin layer of water on the surface of the biomaterial within a few nanoseconds. Then, adhesion proteins are deposited, these are peptides with a specific structure, containing the RGD group – the arginine-glycine-aspartic acid combination.[163,164] Proteins are deposited on the implant surface within seconds to hours. Depending on the surface properties of the biomaterial, proteins settle in different amounts, densities and conformations.[165] The cells of the tissue then recognise the attached proteins, and cell-protein interactions are formed, which last from a few minutes to several days. Cell growth and integration, the final step, enables tissue formation on the scaffold surface.[157,158]

In addition to the surface properties, the scaffold topography and porosity have a significant impact on cell adhesion. The rough structure increases the contact area between the implant and the surrounding tissue and allows cells to settle. Osteoblast differentiation is supported in particular by nanosized roughness.[104,166]The scaffold should be characterised by open porosity, which enables cell migration and colonisation of pores. Open porosity supports the process of vascularisation of the resulting tissue.[160]Cell adhesion is significantly affected by the surface energy of the biomaterial. High surface energy enables very good wettability and

adhesion. Osteoblasts are more likely to settle, differentiate faster and multiply on a surface with higher surface energy.[167–169]

Interaction with groups in chitosan

The biomaterial can interact with the body's cells in different ways. Significant surface features that influence this are surface properties such as hydrophilicity, the presence of functional groups or surface charge.[104,130]

Functional groups on the surface of the biomaterial significantly affect the adhesion of bone cells. The binding of osteoblasts to surfaces with different functional groups was investigated. The hydroxyl–OH and amino–NH_2 groups present in chitosan supported the adhesion and differentiation of stem cells into osteoblasts better than the carboxyl –COOH and methyl–CH_3groups.[170] Stem cell subsidence was most strongly supported by the amino group. Depending on the functional groups, the shape of the cells may also change. Flattened cells were preferred for the –NH_2 group, and spherical cells for –COOH.[171] The surfaces with the amino group also promoted and maintained the process of osteogenesis, both under normal and osteogenesis-promoting conditions.[172]

The wettability of the biomaterial affects the adhesion of cells. Good wettability facilitates the process of proteins settling on the surface of the material and, as a result, cell adhesion. A greater number of adhesive proteins, and a smaller number of hydrophobic proteins that do not participate in the process, were deposited on the hydrophilic surfaces.[163,173]

Proteins settle differently on the positively charged surface than on the negatively charged one. Amino groups of the CS promote the deposition of negatively charged proteins and cell membranes, proliferation and differentiation, which results from electrostatic interactions.[159]

Chitosan with a higher degree of deacetylation provides better conditions for osteoblast subsidence, but to a lesser extent supports the secretion of osteoprotegerin compared to CS with a lower degree of deacetylation.[161,174,175]

Secreted cellular metabolites

There are several types of cells in bone: osteoblasts, osteocytes, osteoclasts, and there are also bone lining cells.[176]Osteoblasts are mature, metabolically active bone-building cells that are found at the surface of the bone. The task of osteoblasts is to secrete compounds that make up the intercellular substance.[177,178] The most numerous cells in bone tissue (over 90%[179]) are bone cells – osteocytes. They arise from transformed osteoblasts trapped in the bone matrix, in the bone cavities. Osteocytes form a communication network of bone cells, thanks to which they regulate bone tissue homeostasis. Signals are sent via cleft junctions, chemical secretion, or direct dendrite association.[180–182] The bone lining cells are osteoblasts that have not undergone apoptosis or developed into osteocytes. They perform functions related to bone remodelling.[176,183] The last group of cells are multinucleated osteoclasts, whose task is to maintain the calcium balance in the tissue and to secrete compounds that break down the bone.[184–187]

The living cells of the body conduct metabolic processes to produce the energy necessary for life from carbohydrates, proteins and fats. For this, the energy carrier adenosine-5'-triphosphate (ATP), produced in various processes, is most often used. Glucose is the main source of energy in mesenchymal stem cells, which is converted into pyruvic acid and ATP in the process of glycolysis. Osteoblasts obtain ATP through glycolysis and the Krebs cycle, with predominance of the former process. The Krebs cycle is used mainly in the period of higher energy demand, during bone formation. In addition to glucose, osteoblasts obtain ATP from the transformation of amino acids (in particular glutamine) and fats. Osteocyte metabolism is not a well understood process. Osteoclasts generate energy in the processes of glycolysis and the Krebs cycle.[188]

The extracellular matrix is mostly produced by osteoblasts. Osteocytes contribute to a lesser extent to the production of matrix, which is due to their structure – mature osteocytes do not have many organelles responsible for secretion.[182] ECM consists mainly of collagen (90%) and non-collagen proteins (10%). The vast majority of collagen is type I collagen, however, type III and V are also found in the ECM. The non-

collagenous organic part consists of proteoglycans, osteocalcin (also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP)), glycoproteins and Small Integrin-Binding LIgand N-linked Glycoproteins (SIBLINGs).[189–191]Proteoglycans are proteins with which saccharide glycosaminoglycans (GAGs) are linked. They are mainly secreted by osteoblasts, e.g. biglikan[192] or decorin.[193]Osteocalcin is secreted mainly by mature osteoblasts, but also by osteocytes.[194] SIBLINGs are small hydrophilic proteins that contain the same set of amino acids (Arg-Gly-Asp) found in the bone matrix. These compounds are mainly produced by osteocytes (Matrix Extracellular Phosphoglycoprotein[195]), but to a lesser extent also by osteoblasts (osteopontin, Dentin Matrix Protein-1[196,197]).[198]

Osteoclasts are capable of secreting H^+–adenosine triphosphate responsible for the dissolution and cathepsin K protease responsible for dissolving the demineralised organic part.[199,200]

Chitosan has a positive effect on the metabolic behaviour of cells. In vitro studies of MCF-7 cells on chitosan scaffolds were performed. The metabolism of cells in such a system is very similar to that in tissue, which proves the positive effect of the chitosan matrix on the vital processes of cells.[201] Similarly, for dental pulp stromal cells, CS has a positive effect on metabolism and proliferation.[202]

CONCLUSION

Chitosan, a chitin derivative, is a unique polysaccharide. It is obtained in the process of deacetylation of chitin, most often derived from the shells of sea crustaceans. It is a readily used compound in tissue engineering due to its similarity to the glycosaminoglycans present in the body, biocompatibility, bioactivity, non-toxicity, antibacterial properties and non-toxic degradation products that may occur under the influence of human enzymes. Amine groups of chitosan improve the surface properties and provide better adhesion of bone cells. CS can be used in the production of cell scaffolds for bone regeneration as a direct substrate or component of a composite. The cationic nature of the polysaccharide also enables the formation of polyelectrolyte complexes with other natural polymers. It can also be used for surface modification to improve the properties of the scaffold.

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Notes

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ABBREVIATIONS

Alg, alginate;

ATP, adenosine triphosphate;

BCNs, bacterial cellulose nanocrystals;

BGLAP, bone gamma-carboxyglutamic acid-containing protein;

CHAP, carbonated hydroxyapatite;

CI, chitin;

Coll, collagen;

CS, chitosan;

CSA, chondroitin sulfate;

DSC, differential scanning calorimetry;

ECM, extracellular matrix;

FTIR, fourier transform infrared spectroscopy;

GAGs, glycosaminoglycans;

Ge, gelatin;

HA, hyaluronic acid

HAP, hydroxyapatite;

MMT, montmorillonite;

nBG, nanobioglass;

nHAP, nano hydroxyapatite;

nPP, nano-pearl powder;

$nSiO_2$, nano-silica;

OMMT, organomontmorillonite;

Pc, pectin;

PCL, polycaprolactone

PCS, phosphorylated chitosan;

PEC, polyelectrolyte complex;

PGS, polyglycerol sebacate;

PLLA, poly-L-lactide;

PVA, poly(vinyl alcohol);

SIBLINGs, Small Integrin-Binding LIgand N-linked Glycoproteins;

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Figure Legends Figure 1. Chitin (a) and chitosan (b). Figure 2. Production of chitosan. Figure 3. Cell adhesion to biomaterial. After biomaterial implementation thin water layer is formed on the surface, followed by protein adhesion. Cells recognise proteins and adhere to the biomaterial, allowing the tissue to regenerate.**Table Legends**Table 1. Chitosan solubility. Table 2. Examples of polyelectrolite complexes with chitosan in tissue engineering Table 3. Examples of chitosan composites in bone tissue engineering

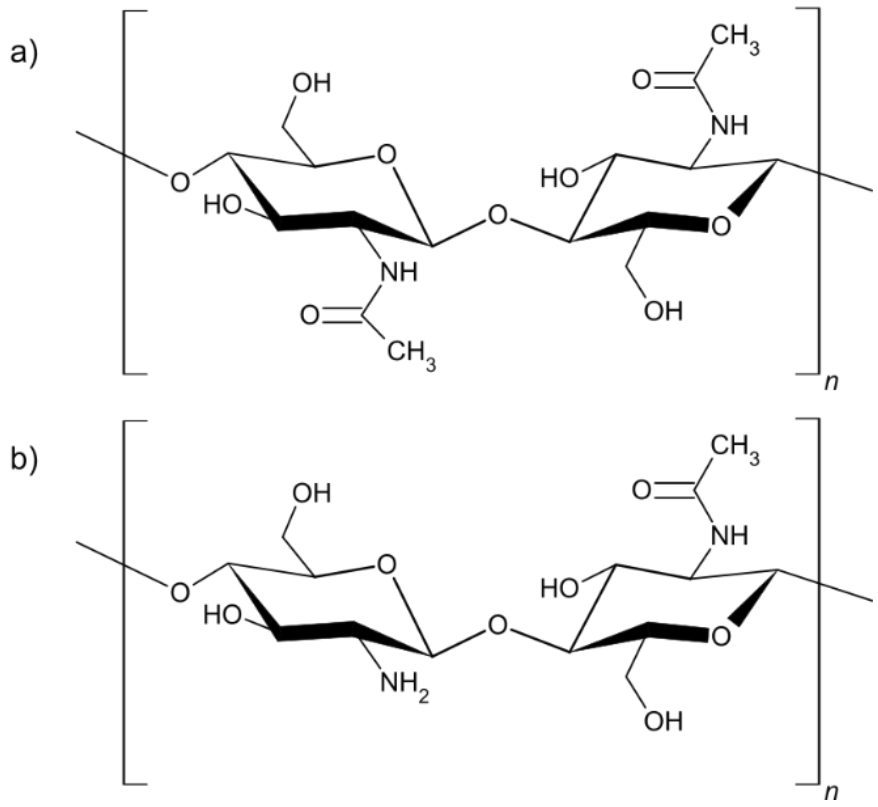

Figure 1. Chitin (a) and chitosan (b).

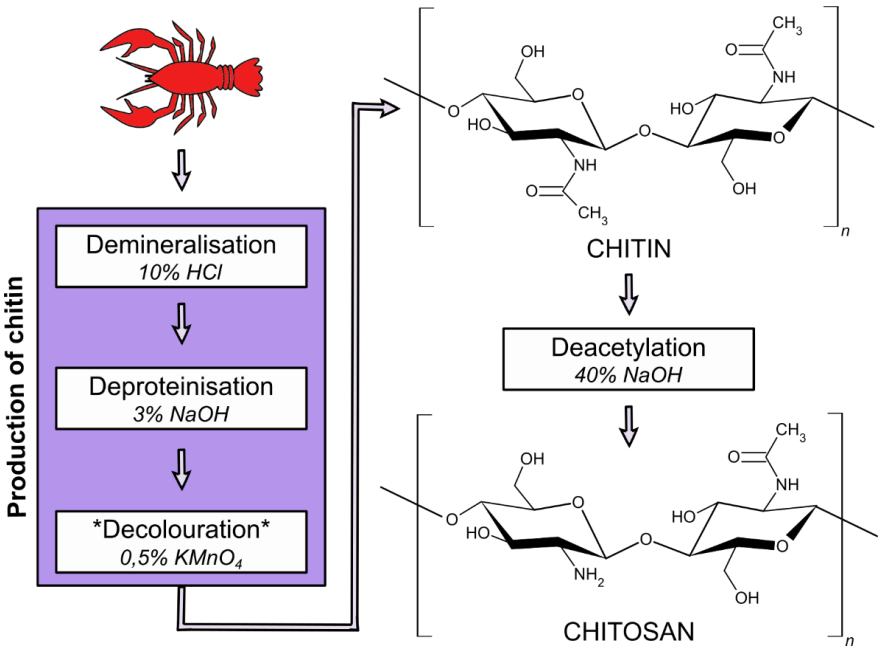

Figure 2. Production of chitosan.

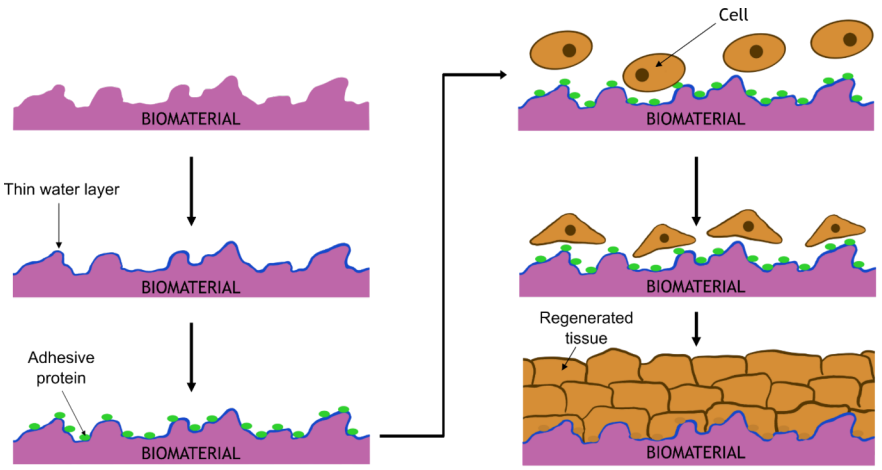

Figure 3. Cell adhesion to biomaterial. After biomaterial implementation thin water layer is formed on the surface, followed by protein adhesion. Cells recognise proteins and adhere to the biomaterial, allowing the tissue to regenerate.

Table 1. Chitosan solubility.[30]

Solvent	**Solubility**
Deionised water	-
2% aqueous acetic acid solution	soluble
Methanol	-
Ethanol	-

Solvent	**Solubility**
Isopropanol	-
Toluene	-
N-Methyl-2-Pyrrolidone (NMP)	-
Methyl ethyl ketone (MEK)	-
Tetrahydrofuran (THF)	-
Dimethyl formamide (DMF)	-
Dimethyl sulfoxide (DMSO)	-

Table 2. Examples of polyelectrolite complexes with chitosan in tissue engineering

Polycation	Polyanion	Abbreviation	Type	Referene
Chitosan	Alginate	CS/Alg	scaffolds	[128–132]
Chitosan	Carboxymethylcellulose	CS/CMC	scaffolds	[133]
Chitosan	Chondroitin sulfate / hyaluronic acid / nano-hydroxyapatite	CS/CSA/HA/nHAP	scaffolds	[134]
Chitosan	Gelatin / hydroxyapatite	CS/Ge/HAP	scaffolds	[135]
Chitosan	Hyaluronic Acid	CS/HA	nanoparticles	[47]
Chitosan	Pectin	CS/Pc	scaffolds	[49,136]
Chitosan	Phosphorylated chitosan	CS/PCS	sponges	[137]

Table 3. Examples of chitosan composites in bone tissue engineering

Composite compounds	Abbreviation	Type	Source
Chitosan + hyaluronic acid	CS+HA	Porous hydrogel	[146]
Alginate + bacterial cellulose nanocrystals + chitosan + gelatin	Alg+BCNs+CS+GT	Porous scaffolds	[147]
Polycaprolactone + chitosan	PCL+CS	Monolayer scaffolds	[148]
Chitosan + organomontmorillonite + hydroxyapatite + ZrO_2	CS+OMMT+HAP+ZrO_2	Films	[149]
poly(vinyl alcohol) + chitosan + carbonated hydroxyapatite	PVA+CS+CHAP	Nanofibrous scaffolds	[150]
β-cyclodextrin + nano-hydroxyapatite + chitosan	nHAP+β-CD+CS	Nanoparticles	[151]
Chitosan + hyaluronic acid + nano-pearl powder	CS/HA+nPP	Porous scaffolds	[152]
Polyglycerol sebacate + chitosan + gelatin	PGS+CS+Ge	Nanofibrous scaffolds	[153]
Zeolite A + chitosan	-	Porous scaffolds	[154]
Gelatin + chitosan + nanobioglass	Ge+CS+nBG	Porous scaffolds	[155]

Composite compounds	Abbreviation	Type	Source
Chitosan+poly(vinyl alcohol)+TiO_2	CS+PVA+TiO_2	Films	[156]