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Biomimetic scaffolds based on chitosan in bone regeneration. A review

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Chitosan (CS) is a polysaccharide readily used in tissue engineering due to its properties: similarity to the glycosaminoglycans present in the body, biocompatibility, non-toxicity, antibacterial character and owing to the fact that its degradation that may occur under the influence of human enzymes generates non-toxic products. Applications in tissue engineering include using CS to produce artificial scaffolds for bone regeneration that provide an attachment site for cells during regeneration processes. Chitosan can be used to prepare scaffolds exclusively from this polysaccharide, composites or polyelectrolyte complexes. A popular solution for improving the surface properties and, as a result enhancing cell-biomaterial interactions, is to coat the scaffold with layers of chitosan. The article focuses on a polysaccharide of natural origin – chitosan (CS) and its application in scaffolds in tissue engineering. The last part of the review focuses on bone tissue and interactions between cells and chitosan after implantation of a scaffold and how chitosan's structure affects bone cell adhesion and life processes.

Keywords: chitosan, scaffolds, biocompatibility, bone regeneration

1. INTRODUCTION

Bone lesions are common injuries. In some cases, the bone can regenerate a sufficiently small defect size (Alonzo et al., 2021; Kanczler et al., 2020). There are also injuries, so-called critical defects, that cannot regenerate spontaneously and require special treatment. It is assumed that the size of such lesions is approx. 1–2 cm and more than 50% of the bone volume is affected (Schemitsch, 2017). Regeneration of such defects is complicated, difficult to control; so far, it has involved autologous bone transplant surgery (Giannoudis et al., 2005; Kozusko et al., 2018; Pape et al., 2010). Alternative to conventional transplants, scaffolds made from biomaterials can be used. Scaffolds will provide a place for attachment for new bone cells and form the basis for the reconstruction process (Deb et al., 2018; Preethi Soundarya et al., 2018).

Biomaterials are the primary materials used in tissue engineering. They must be biocompatible, effective, and sterilisable (Budnicka et al., 2020; Hudecki et al., 2019). The use of biomimetic materials is currently popular. They are bioresorbable and designed to stimulate the growth and proliferation of cells on the material's surface (Hench and Polak, 2002; Navarro et al., 2008). The literature review aimed to study the popular polysaccharide of natural origin – chitosan, its properties, behaviour in contact with body cells, and applications in bone tissue engineering.

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2. CHITOSAN

Chitosan (CS) is a linear cationic polymer, a copolymer composed of N–acetyl–D–glucosamine and D–glucosamine units linked by β 1,4 glycosidic bonds (Fig. 1).

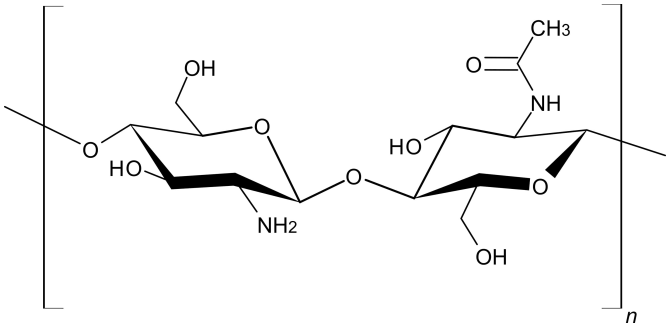

Fig. 1. Chitosan

2.1. Preparation and chemical structure

The source of chitosan is chitin (CI) – the second most common polysaccharide in nature (Islam et al., 2020). CI is one of the main building blocks of arthropod skeletons and fungal cell walls (Bastiaens et al., 2019; Lalzawmliana et al., 2019). Industrial chitin is most often obtained from food industry waste, crustaceans or sea molluscs (shrimps, lobsters, crabs, squid, mussels) (Bakshi et al., 2020; Wang et al., 2020b; Yadav et al., 2019; Zargar et al., 2015) although it may also be of fungal origin (Pochanavanich and Suntornsuk, 2002).

Due to its low solubility in popular solvents, chitin is obtained in a multi-stage extraction process of chemical purification (Fig. 2). The dried shells of crustaceans are demineralised by acid (commonly 10% aqueous HCl solution) to remove minerals, especially calcium (Kou et al., 2021; Younes and Rinaudo, 2015). In the next, deproteinisation step, the residues are treated with alkalines, e.g. NaOH solution, to remove proteins (Kumari and Kishor, 2020). The obtained chitin of a pinkish colour can be subjected to a decolouration process under the influence of an oxidant, e.g. $KMnO_4$, H_2O_2 (Islam et al., 2020; Younes and Rinaudo, 2015). It is also possible to purify chitin biologically with the use of enzymes – proteases

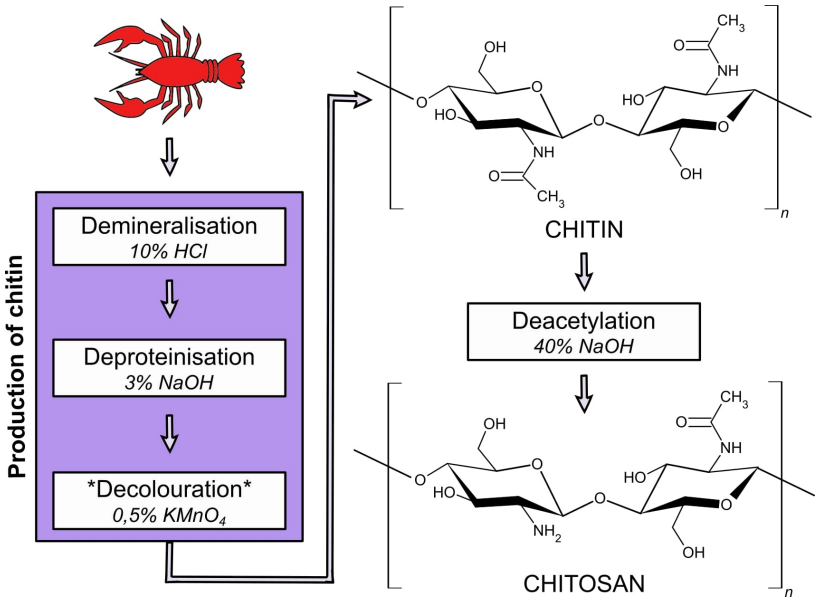

Fig. 2. Production of chitosan

(Arbia et al., 2013; Valdez-Peña et al., 2010; Younes et al., 2014) or microbes in the fermentation process (Doan et al., 2019).

The purified chitin is subjected to a deacetylation process (Fig. 2). To this end, the CI is treated with a concentrated base, most often 40% NaOH (Kou et al., 2021). The chitosan thus obtained is in the form of a white powder (Islam et al., 2020). Random acetyl groups are removed in the deacetylation process, thus replacing some N–acetyl–D–glucosamine with D–glucosamine units. The presence of the latter groups depends on the degree of deacetylation (DD) used to characterise the CS. The more deacetylated the CS, the fewer N–acetyl–D–glucosamine groups. The value of the DD of chitosan is in the range of 50–95% (Aravamudhan et al., 2014; Wang et al., 2020b).

2.2. Physicochemical properties

Chitosan is insoluble in water or popular organic solvents (including methanol, ethanol, isopropanol, toluene). However, it dissolves in dilute aqueous acid solutions, e.g. hydrochloric acid, acetic acid, formic acid and citric acid (Grant and Allen, 2006; Lu et al., 2004; Tsai et al., 2009). Due to the presence of free amino groups that can protonate at a pH below 6 (Chicatun et al., 2017; Leedy et al., 2011), CS forms with acids water-soluble salts (Ravi Kumar et al., 2004). The solubility of chitosan depends on the degree of deacetylation (DD) and the molecular weight of the polysaccharide (Madureira et al., 2015; Wang et al., 2020b). Due to its positive charge, chitosan can form complexes with negatively charged compounds, e.g. polylactide (Wan et al., 2007), poly(glutamic acid) (Antunes et al., 2011), DNA (Maurstad et al., 2007; Strand et al., 2005), collagen (Kaczmarek et al., 2018), alginates (Park et al., 2019; Phoeung et al., 2017; Wang et al., 2010), hyaluronic acid (Gennari et al., 2019; Lallana et al., 2017; Oyarzun-Ampuero et al., 2009), pectin (Bombaldi de Souza et al., 2019; Pandey et al., 2013) and many others. The presence of hydrophobic acetyl groups and hydrophilic amine and hydroxyl groups ensures the amphiphilic nature of the chitosan (Islam et al., 2020). The amine groups that are responsible for the cationic nature of the polymer result in high surface tension of aqueous solutions of this polysaccharide (Xu and Yang, 2014).

The structure of chitosan, based on D–glucosamine and N–acetyl–D–glucosamine units, is similar to the structure of naturally occurring glycosaminoglycans (GAG). GAGs build the extracellular matrix and perform various functions in organisms, so it can be expected that chitosan can also easily interact with animal cells thanks to its GAG-like structure (Chicatun et al., 2017; Preethi Soundarya et al., 2018). CS 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) (Islam et al., 2020; Luo et al., 2008; Wu et al., 2014), it inhibits the growth of neoplastic cells (Shi et al., 2017; Yuan et al., 2019) and promotes wound healing (Abd El-Hack et al., 2020; Duan et al., 2019; Kim and Shin, 2013; Kucharska et al., 2019). Chitosan also has antibacterial and antifungal properties (against both gram-positive (Li et al., 2020b; Raafat et al., 2008) and gram-negative bacteria (Helander et al., 2001; Li et al., 2020b)). These properties are probably due to the interaction between the positively charged polymer and negatively charged lipopolysaccharides or proteins in microbial membranes (Kucharska et al., 2019). 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 (Ahmad et al., 2020; Kucharska et al., 2019; Kumar et al., 2019).

2.3. Chitosan degradation

Scaffolds must be sterilised before implementation. Chemical or radiation sterilisation is commonly used. Chitosan is a natural polymer that decomposes under the influence of both chemical and physical factors, and such changes may affect the properties of the polymer and, consequently, the chitosan-containing medical device (Pandit et al., 2021; Petrov et al., 2016).

Chemical factors include depolymerisation under the influence of strong oxidants (Ma et al., 2014) or free radicals, (Hsu et al., 2002) popularly used in the sterilisation process. The products are chitosan oligosaccharides with various, low molecular weights (Yan et al., 2020). Chemical degradation is fast but poorly controlled (random cutting of the polymer chain) (Je and Kim, 2012; Tsao et al., 2011; Yuan et al., 2019).

It was examined that the CS structure changes under the influence of gamma radiation used in radiation sterilisation. A decrease in molecular weight of polysaccharide is observed after exposure to radiation of 15 kGy, the effect is stronger with increasing radiation dosage (San Juan et al., 2012). Contrary to chemical depolymerisation, cuts occur more frequently inside the polymer, (Lim et al., 1998) and the obtained oligosaccharides are characterised by a narrower size distribution (Yan et al., 2020).

2.4. Metabolism in living organisms

A biodegradable scaffold used in bone regeneration should degrade in the body into well-known, non-toxic products that can be excreted 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's characteristics (their age, chronic diseases), but on average in bone regeneration, it should take from 3 to 9 months (Budnicka et al., 2018).

Glycosidic bonds in chitosan can undergo non-enzymatic hydrolysis, but a process occurs very slowly (Jennings, 2017; Kim et al., 2008). Enzymatic degradation of chitosan can occur under the influence of specific or non-specific enzymes (Je and Kim, 2012). The specific enzymes in the human body that hydrolyse the β-1,4-glycosidic bonds present in chitosan are chitinases (Sørbotten et al., 2005). Non-specific enzymes are, for example, human pancreatic lipase or lysozyme (Halim et al., 2012; Nawrotek et al., 2020). Chitosan also degrades under the influence of enzymes produced by bacteria present in the colon (Guarino et al., 2015). The rate of chitosan degradation depends on properties of the polysaccharide, such as molecular weight, degree of deacetylation, crystallinity (Zhang and Neau, 2002). Products of the process are non-toxic oligosaccharides of various molecular sizes, which can be easily removed from the body in urine (Onishi and Machida, 1999; Szymańska and Winnicka, 2015). Chitosan does not elicit a long-lasting inflammatory or immune response (Abarrategi et al., 2010; El-Sayed et al., 2021; Kim et al., 2011). The inflammation observed after introducing chitosan depends on the degree of deacetylation of the polysaccharide: chitosan with a lower DD induced a stronger leukocyte response (Barbosa et al., 2010). The degradation of scaffolds made of chitosan with similar DD but in different forms: microspheres (Mi et al., 2002), hydrogel (Moura et al., 2017), and 3D porous scaffold, occurs at different times (Qasim et al., 2017).

It has been noted that chitosan can interact with blood cells due to its cationic nature. In the presence of chitosan, blood tends to coagulate and form clots on the surface of chitosan (Hirano et al., 1987). 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 (Hirano et al., 1991). In vivo degradation studies of chitosan-poly-L-lactide composites in rats showed that the polysaccharide did not cause an unexpected, negative response, and no blood clots were detected after a week (Guo et al., 2018).

3. CELL SCAFFOLDS

Tissue engineering is looking for solutions to replace the need for autographic or allographic transplants. One of the proposed solutions is the use of scaffolds that mimic the fundamental roles of tissues and enable their regeneration (Chan and Leong, 2008; Salgado et al., 2013).

Such scaffolds is usually three-dimensional, porous structures made of biomaterial. The main task of scaffold is to support the regenerating tissue by providing an artificial matrix on the surface of which cells can settle (Deb et al., 2018). Scaffolds should imitate the tissue for which they are used to regenerate, its structure and properties (O'Brien, 2011). The implant must not cause an adverse 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 (Middleton and Tipton, 2000).

3.1. Scaffolds made of chitosan alone

Chitosan supports cell adhesion and proliferation and thus tissue regeneration. CS can be easily modelled to a specific shape and create porous structures with open pores (García Cruz et al., 2010). It is suitable as a material for 3D printing (Sadeghianmaryan et al., 2020). These features make chitosan a potential candidate for scaffold material.

Production conditions significantly affect the porosity, pore size or mechanical properties related to the compressive modulus of scaffolds (Reys et al., 2017). Scaffolds made of chitosan with a higher degree of deacetylation are characterised by higher mechanical resistance, slower degradation time and lower absorbability of the scaffold (Thein-Han and Kitiyanant, 2007). Also, the scaffold's porosity significantly influences its mechanical properties (Sadeghianmaryan et al., 2020); 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 (Xu et al., 2017).

In vitro and in vivo studies are conducted on chitosan scaffolds to treat damage to various tissues. The interactions and influence of CS on cells are investigated, among others, spinal cord (Kim et al., 2011), bones, cartilage (Abarrategi et al., 2010), tendons (Chen et al., 2018a) and skin (Intini et al., 2018). The use of chitosan in the treatment of the spinal cord has been studied. After the implant insertion into the meninges or directly into the spinal cord for 6 or 12 months, the host (rat) cell response was low, which indicates the inert nature of chitosan and the possibility of using the material in long-term therapies (Kim et al., 2011). In vivo studies suggest that the cellular response depends on the properties of the chitosan used, and chitosan scaffolds promote the shaping of the subchondral bone (Abarrategi et al., 2010). High DD (DD 88% and 95% tested) supports cell proliferation on the surface of the chitosan scaffold (Thein-Han and Kitiyanant, 2007).

Active layers can be applied to the scaffolds to improve the interaction at the biomaterial-tissue interface (Budnicka et al., 2018). A promising method is to subject scaffolds to a biomineralisation process. Calcium and phosphate ions from body fluid settle down on a scaffold, thus creating hydroxyapatite layers imitating bone (Saravanan et al., 2018). After modification, the scaffolds are characterised by greater rigidity and smaller pore sizes, yet sufficiently large to allow unhindered migration of bone cells (Dash et al., 2017). 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 (Aday and Gümüşderelioğlu, 2009; Manjubala et al., 2008).

3.2. 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, and properties supporting bone regeneration, such as osteoconductivity (Di Martino et al., 2005; Roach et al., 2007). 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 (Gallyamov et al., 2018; Kumari et al., 2021). Surface modification significantly influences the mechanical properties of the scaffold. As chitosan concentration in the layer increases, the compression strength and the compression modulus factor increase, while scaffolding crystallinity decreases (Poddar et al., 2021). The increase in the first two is likely related to the rise in the number of amine groups that support the scaffold's material, polycaprolactone (PCL), and improve the strength of the scaffold. The decrease in crystallinity results from hydrogen bonds between PCL and chitosan (Poddar et al., 2021).

It is commonly known that the hydrophilicity/hydrophobicity of the scaffold affects cell adhesion and proliferation. Polylactide (PLA) is a biocompatible and bioresorbable polymer. It is, however, a hydrophobic polymer that does not promote cell adhesion. To increase the hydrophilicity of the polymer, the PLA surface can be modified by applying a layer of chitosan (Zhu et al., 2002). Modification of poly-L-lactide surface with chitosan also improves mechanical properties of the scaffold, as well as enhances adhesion, differentiation, activity, and morphology of chondrocyte cells (Ma et al., 2002) and mouse bone marrow stromal cells (mBMSCs) compared to the unmodified structure, which accelerates the bone regeneration process (Chen et al., 2018b; Jiao et al., 2007). 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 supported more significant bone tissue growth with higher density than the unmodified ones. This confirms that chitosan-coated polylactide scaffolds are sustainable for bone regeneration (Chen et al., 2018b) and modifying the scaffold surface with chitosan significantly improves osteoblast adhesion and proliferation (He et al., 2021). Chitosan coating's antimicrobial properties were tested on gram-negative strains of *E. coli* (Al-Nabulsi et al., 2020; Lin et al., 2021) and gram-positive *Staphylococcus aureus* (Foss et al., 2015). For both groups, the use of chitosan layers reduced bacterial settling.

In addition to applying chitosan layers, mixtures of chitosan and other compounds are also used to improve specific properties. Using 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 (Lin et al., 2021). Incorporating Ca^{2+} and PO_4^{3-} to chitosan-modified PLLA scaffolds induces hydroxyapatite formation, thus improving osteoconductive properties (Mano et al., 2008). Antibiotics can also be incorporated into the chitosan layer. The compounds bind through weak intermolecular interactions, allowing the easy release of the antibiotic and, consequently, the fight against bacteria (Zarghami et al., 2021).

3.3. Complexes with other natural polymers

Due to the cationic nature of the polymer, CS can spontaneously form stable polyelectrolyte complexes (PEC) with negatively charged structures such as natural polymers. In a solution, permanent electrostatic interactions are formed between the cationic amino group of chitosan and the negatively charged group of the polyanion (Kulkarni et al., 2016; Wu et al., 2020). PEC formation can be investigated with FTIR (N–H, C=O bond shift study), DSC differential scanning calorimetry (melting point shift), or by examining changes in zeta potential (Lal et al., 2017; Lallana et al., 2017). The wide range of properties, non-toxicity, biodegradability, and biocompatibility of PEC ensure a broad application of the complexes, especially in tissue engineering (Potaś et al., 2020). Examples of PEC are shown in Table 1.

The size of the complex particles depends on the molecular weight and concentration or addition order of polymers used. The larger the polymer, the thinner the packing and the larger the size of the PEC (Strand et al., 2005). The diameters of the chitosan with pectin complexes (CS/Pc) nanoparticles varied depending on the order of addition and concentration of polymers. Large particles (460 nm) were formed at low polymer concentrations when chitosan was added first; the inverse was true at high concentrations

Table 1. Examples of polyelectrolyte complexes with chitosan in tissue engineering

Polycation	Polyanion	Abbrv.	Characteristics	Reference
Chitosan	Alginate	CS/Alg	3D scaffolds with increased compressive modulus and yield strength, good osteoblast affinity	Li et al. (2005)
			3D scaffolds coated with HAP, increased mechanical properties, good osteoblast affinity	Patil et al. (2017)
			Nontoxic biomaterial, anti-inflammatory properties	Hardy et al. (2018)
			PEC fibres as a controlled protein encapsulation system	Liao et al. (2005)
	Carboxymethylcellulose	CS/CMC	3D scaffolds composed with calcium phosphate, increased compressive strength and compressive modulus, increased osteoblast proliferation	Unagolla et al. (2018)
	Chondroitin sulfate / hyaluronic acid / nano-hydroxyapatite	CS/CSA/ HA/nHAP	Porous scaffolds with improved mechanical properties, good biocompatibility that aids osteoblast proliferation	Hu et al. (2017)
	Gelatin / hydroxyapatite	CS/Ge/ HAP	3D porous scaffolds inducing osteogenic differentiation	Sellgren and Ma (2012)
	Pectin	CS/Pc	Porous scaffolds with improved elastic moduli, non-toxic products of degradation	Bombaldi de Souza et al. (2019)
			3D porous scaffolds, osteoblast affinity	Coimbra et al. (2011)
	Phosphorylated chitosan	CS/PCS	3D porous scaffolds, good osteoblast affinity	Li et al. (2007)

(Birch and Schiffman, 2014). 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 resulting complexes' size, charge, and solubility (de Vasconcelos et al., 2006). The stability of PEC is independent of particle size, but it is dependent on the pH of the solution (Birch and Schiffman, 2014). Molecules or ions, other than the polymers forming the complex, present in the solution affect the ease of forming complexes, their stability, and their properties. An example of such a compound is sodium lauryl sulphate surfactant, which increases the surface roughness and the thickness of the chitosan/alginate (CS/Alg) (de Vasconcelos et al., 2006; Kaygusuz et al., 2017).

The stability of scaffolds made of polyelectrolyte complexes depends on the properties of polymers, such as molecular weight or, in the case of chitosan, degree of deacetylation (Bombaldi de Souza et al., 2019). Compared to chitosan-only scaffolds incorporating alginate caused an almost threefold increase in the compressive modulus and yield strength (Li et al., 2005). It is because the electrostatic interaction between the chitosan and polyanion increases the stability of the scaffolds obtained compared to chitosan-only scaffolds (Park et al., 2013). For different polyanions, mechanical properties differ (Baghaei et al., 2021),

for example, the elastic modulus of scaffolds containing alginate are lower than for the pectin-containing ones (Bombaldi de Souza et al., 2019). The same dependence is observed in the degradation process: chitosan complex with alginate degrades faster than another polyanion. It can be attributed to differences in the crosslinking of both formulations (Bombaldi de Souza et al., 2019).

Introduction of hydroxyapatite (HAP) into PEC scaffolds improves mechanical strength. Carboxyl groups of polyanions are crosslinked by Ca^{2+} ions introduced in the biomineralisation process. As a result, scaffolds' mechanical properties and stability increase while water uptake and degradation rate get lower (Bombaldi de Souza et al., 2019; Kaczmarek et al., 2018; Patil et al., 2017). The increase in mechanical properties may also be due to a decrease in porosity of scaffolds as a result of pore filling by the HAP particles (Patil et al., 2017).

The polyelectrolyte complexes of chitosan with biogenic polysaccharides are non-toxic, show no inflammatory effects, support cell adhesion, as well as osteoblast proliferation and angiogenesis (Hardy et al., 2018; Kaczmarek et al., 2018; Li et al., 2005; Patil et al., 2017). However, some sources report the CS/Alg surface as unfavourable for cell adhesion (Phoeung et al., 2017). Incorporating HAP into scaffolds improves interactions with cells (Kaczmarek et al., 2018; Unagolla et al., 2018).

3.4. 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 cannot carry the required loads (Islam et al., 2020). To change the properties of the scaffold, a new compound can be introduced into the starting composition to obtain a composite (Unnithan et al., 2017). The use of the composite influences morphology, mechanical properties, porosity, swelling of the scaffolds, cellular interactions and degradation processes. Examples of such composites are presented in Table 2.

The morphology of the scaffolds can be controlled by changing the concentration of the polymers used. For the poly-L-lactide + chitosan (PLLA+CS) scaffold, the higher the chitosan concentration in the starting solution, the more chitosan was deposited on the pore surface, making the pores surface more jagged compared to polylactide systems alone (Mano et al., 2008). This is a desirable phenomenon because of the increase in scaffold surface area and, as a result, the increase in the space available for cells to attach (Kara et al., 2019).

The use of composites has a significant influence on mechanical properties. To increase the compressive strength of macrospheres made of chitosan, montmorillonite (MMT) and hydroxyapatite (HAP) can be added (Vyas et al., 2017). Inorganic particles act as physical crosslinking sites, increasing the polymer network's stability and load-bearing capacity (Cao et al., 2015). The swelling of scaffolds is an essential process for proper tissue regeneration. It promotes protein adsorption and the diffusion of nutrients and gases. Increasing the scaffold's surface area also aids cell adhesion (Ali et al., 2022). Changes in swelling of composites are caused by the introduction of additional hydrophilic or hydrophobic groups (Li et al., 2021) The composite made of poly(vinyl alcohol) and chitosan (PVA+CS) showed lower swelling than in the case when carbonated hydroxyapatite was introduced (Januariyasa et al., 2020). Swelling ratios of scaffolds can be decreased by crosslinking (Suo et al., 2021) and binding or blocking hydrophilic groups (Scalera et al., 2021; Vyas et al., 2017). Interestingly, introducing montmorillonite (MMT) to chitosan-only scaffolds reduced swelling by forming a barrier that inhibited interactions between water particles and polymer (Vyas et al., 2017).

Increased hydrophilic properties of composites accelerate non-enzymatic degradation processes. Introducing hydrophilic compounds like chitosan into hydrophobic scaffolds accelerates water's diffusion into the interior, making hydrolysis of the hydrophobic polylactide faster (Guo et al., 2018). In composites,

Table 2. Examples of chitosan composites in bone tissue engineering

Composite compounds	Abbrv.	Characteristics	Reference
poly(vinyl alcohol) + chitosan + carbonated hydroxyapatite	PVA+CS+CHAP	Nanofibrous scaffolds supported osteoblasts adsorption and proliferation	Januariyasa et al. (2020)
Chitosan + poly(vinyl alcohol) + TiO_2	CS+PVA+TiO_2	Nanocomposite films, antibacterial properties, good osteoblast affinity	Khan et al. (2020)
Chitosan + organomontmorillonite + hydroxyapatite + ZrO_2	CS+OMMT+ HAP+ZrO_2	Films, antibacterial properties, good osteoblast affinity	Bhowmick et al. (2016)
Chitosan + hyaluronic acid	CS+HA	Porous hydrogel supported cultures of human osteoblasts and osteoclasts	Beşkardeş et al. (2017)
Alginate + bacterial cellulose nanocrystals + chitosan + gelatin	Alg+BCNs+ CS+GT	Porous 3D scaffolds, good osteoblastic cells affinity	Yan et al. (2017)
Polycaprolactone + chitosan	PCL+CS	Monolayer scaffolds, mechanical properties comparable to that of cancellous bone, high elasticity	Thuaksuban et al. (2013)
β-cyclodextrin + nano-hydroxyapatite + chitosan	nHAP+β-CD+CS	Nanoparticles of good osteoblast affinity, antibacterial properties	Shakir et al. (2016)
Chitosan + hyaluronic acid + nano-pearl powder	CS/HA+nPP	Porous 3D scaffolds, increased mechanical properties, supported osteoblasts proliferation and differentiation	Li et al. (2020a)
Zeolite A + chitosan	–	Porous nanocomposite scaffolds supported osteoblasts adhesion and proliferation	Akmammedov et al. (2018)
Gelatin + chitosan + nanobioglass	Ge+CS+nBG	Porous 3D scaffolds, porosity and mechanical properties comparable to that of cancellous bone, good osteoblast affinity	Maji et al. (2016)

other effects might occur that affect the degradation ratio. Such an example is PLLA+CS. When PLLA hydrolyses into lactic acid, it lowers the pH of the environment, which causes the dissolution of the CS, which increases PLLA surface contact with water (Guo et al., 2018).

The introduction of inorganic particles into the composite can have a twofold effect on porosity: either increasing or decreasing it (Cao et al., 2015; Li et al., 2021). The incorporation of MMT made it possible to obtain a structure with well-connected pores of small diameters from a poorly connected network of large pores. It is attributed to interactions between MMT and amino groups of the polymer, resulting in

smaller, more regular pores (Cao et al., 2015). On the other hand, porosity can decrease due to the physical occupation of space by HAP particles deposited in the pores of the scaffold (Li et al., 2021).

Chitosan-hydroxyapatite composite (CS+HAP) scaffolds are eagerly studied. Such systems mimic bone extracellular matrix, show no cytotoxicity and exhibit osteogenic properties (Ali et al., 2022; Danilchenko et al., 2011; Ressler et al., 2022; Zafeiris et al., 2021). In vivo studies show that porous CS+HAP scaffolds undergo almost complete biodegradation and are replaced by regenerating tissue 24 days after the implementation (Danilchenko et al., 2011). Increasing HAP concentrations increases the compressive strengths of the scaffolds (Patil et al., 2022). As mentioned before, mechanical properties can change due to HAP particles filling pores, thus reducing porosity and improving mechanical resistance (Ali et al., 2022; Patil et al., 2017).

HAP can also be introduced in the form of nanoparticles (nHAP), inducing similar effects of improving susceptibility to mineralisation or positively influencing interactions with bone cells (Dan et al., 2016). In addition to hydroxyapatite, other nanoparticles, e.g., $nZrO_2$, $nSiO_2$ or TiO_2, are also used in composites to produce non-toxic, bioactive scaffolds (Bhowmick et al., 2016; Jayakumar et al., 2011; Kavya et al., 2013; Khan et al., 2020; Vaidhyanathan et al., 2021). Chitosan composites exhibit antibacterial properties against both gram-positive and gram-negative bacteria like chitosan itself (Khan et al., 2020).

4. CELLULAR RESPONSE TO CHITOSAN MATERIALS

After scaffold implantation at a damaged area, the body responds with a series of actions that ultimately lead to tissue regeneration. The immune system immediately recognises the implant and triggers appropriate interactions: clot formation, cellular response and tissue reconstruction (Bosco et al., 2012). A thin layer of water forms on the scaffold surface that proteins adhere to. The bone cells then recognise these proteins and settle on the implant. Cell growth and integration enable tissue to regenerate on the scaffold surface (Anselme, 2000; Paital and Dahotre, 2009). Depending on the surface chemistry and implant topography, cells may interact with the material with different intensities.

4.1. Cell adhesion

The term "adhesion" describes two phenomena that occur after the implantation of the scaffold. 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 (Anselme, 2000). Cell attachment to the surface is essential for their proper growth, division and proliferation (Nowacka, 2012). Proteins containing specific Arg-Gly-Asp (RGD) sequences in extracellular matrix (ECM) are necessary to mediate cell adhesion (Ruoslahti, 1996). Cell receptors recognise RGD motifs and aid cell adhesion to other cells or surfaces (Cavalcanti-Adam et al., 2008; Pierschbacher et al., 1994). Adhesion receptors are transmembrane adhesion proteins that connect cells with their environment, used to obtain information from the outside. In addition to integrins, the primary ECM receptors, cadherins, immunoglobulin superfamily (Ig-SF) and selectins are also involved in adhesion processes (Hynes, 1999). In bones, integrins connect osteoblasts or osteoclasts to glycoproteins in ECM such as collagen type I, osteopontin, fibronectin, consequently inducing cell adhesion, migration, growth or proliferation (Klein-Nulend and Bonewald, 2020; Taylor et al., 2022).

Implants are recognised by the organism as foreign bodies and trigger an immediate immune response, leading to the formation of a fibrous layer on the biomaterial's surface (Nowacka, 2012). The body's response mechanism after implantation (Fig. 3) begins with forming a thin layer of water on the surface of the biomaterial within a few nanoseconds (Cavalcanti-Adam et al., 2008). Proteins with the characteristic

RGD motif are deposited on the implant surface, creating a protein layer within seconds to hours (Nowacka, 2012; Scotchford et al., 2002). Depending on the surface properties of the biomaterial, proteins settle in different amounts, densities and conformations (Roach et al., 2007). The cells recognise the attached proteins, and cell-protein interactions are formed, lasting from a few minutes to several days. The final step of cell growth and integration enables tissue formation on the scaffold surface (Bosco et al., 2012; Paital and Dahotre, 2009).

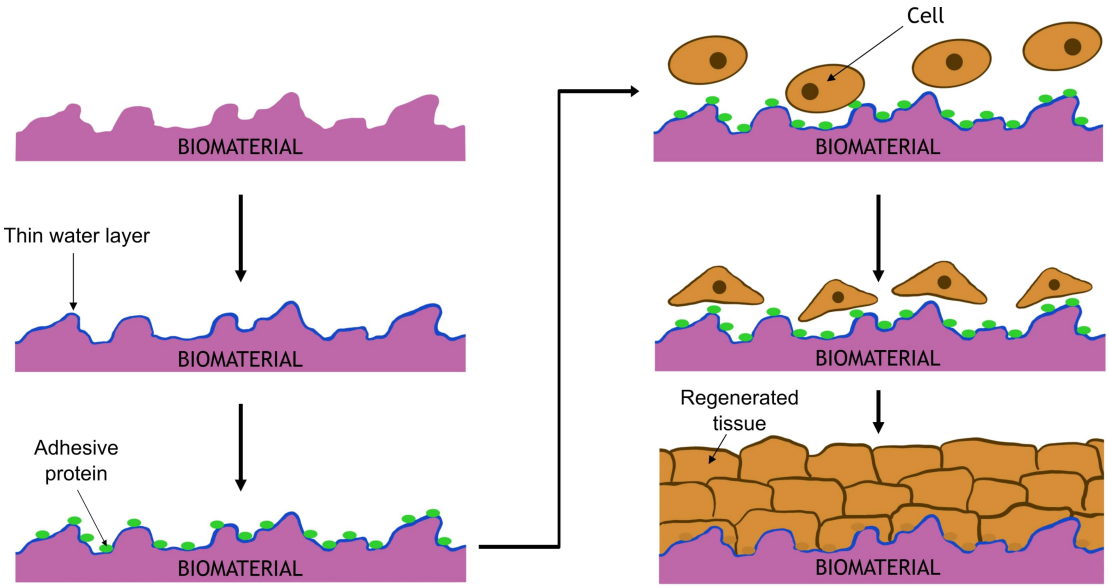

Fig. 3. Cell adhesion to the biomaterial. After biomaterial implementation a thin water layer is formed on the surface, followed by protein adhesion. Cells recognise proteins and adhere to the biomaterial, regenerating tissue

The surface properties of the material control adhesion processes, so modifications with integrin agonists are used to enhance properties that promote cell adhesion, growth and proliferation (Cirillo et al., 2021). Depending on a surface potential, the material can inhibit or improve adhesion (Guette-Marquet et al., 2022). In addition to the surface properties, the scaffold topography and porosity significantly impact 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 (Budnicka et al., 2018; Xiao et al., 2017). The scaffold should be characterised by open porosity, enabling cell migration and colonisation of pores. Open porosity supports the process of vascularisation of the resulting tissue (Gadomska-Gajadhur et al., 2018). Cell adhesion is also significantly affected by the surface energy of the biomaterial. High surface energy enables excellent wettability and adhesion. Osteoblasts are more likely to settle, differentiate faster and multiply on a surface with higher surface energy (Comelles et al., 2010; Tian et al., 2019; Yang et al., 2012).

4.2. Interactions with groups in chitosan

Biomaterials used in tissue engineering should be osteoconductive and/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 (Albrektsson and Johansson, 2001; Gadomska-Gajadhur et al., 2018). Osteoinduction involves triggering a series of processes and reactions that lead to bone regeneration through biomolecular signalling devices. One such process is, for example, cell differentiation (Cao et al., 2020; Habibovic and de Groot, 2007; Porrelli et al., 2021). The nature of a biomaterial is determined by its surface properties such as hydrophilicity, the presence of functional groups or surface charge (Budnicka et al., 2018; Wang et al., 2020a).

Functional groups on the biomaterial surface significantly affect the adhesion processes of bone cells. Researching the binding of osteoblasts to surfaces with different functional groups shows that 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 $–CH_3$ groups (Keselowsky et al., 2005). 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 (Curran et al., 2005). The surfaces with the amino group also promoted and maintained the process of osteogenesis, both under normal and osteogenesis-promoting conditions (Curran et al., 2006). A more significant number of adhesive proteins, and a smaller number of hydrophobic proteins that do not participate in the process, were deposited on the hydrophilic surfaces, which confirms that good wettability facilitates the process of protein deposition on the surface of the material and, as a result, cell adhesion (Nowacka, 2012; Sobieska et al., 2013). 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 (Anselme, 2000). 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 DD (Cao et al., 2020; Govindasamy et al., 2020; Sukul et al., 2021).

4.3. Secreted cellular metabolites

There are several types of cells in bone: osteoblasts, osteocytes, osteoclasts and bone lining cells (Florencio-Silva et al., 2015). Osteoblasts are mature, metabolically active bone-building cells found at the surface of the bone, that are tasked to secrete compounds that make up the intercellular substance (Dirckx et al., 2019; Ducy et al., 2000). The most numerous cells in bone tissue (over 90%) – osteocytes form a communication network of bone cells, which regulate bone tissue homeostasis (Aarden et al., 1994; Bonewald, 2010; Datta et al., 2008; Klein-Nulend and Bonewald, 2020). The bone lining cells are osteoblasts that have not undergone apoptosis or developed into osteocytes. They perform functions related to bone remodelling (Brown et al., 2013; Florencio-Silva et al., 2015). The last group of cells are multinucleated osteoclasts, whose task is to maintain the tissue's calcium balance and secrete compounds that break down the bone (Jang et al., 2009; Kalfas, 2001; Morgan et al., 2013).

The body's living cells conduct metabolic processes to produce the energy using the energy carrier adenosine-5′-triphosphate (ATP), created in various processes. Osteoblasts obtain ATP through glycolysis and the Krebs cycle, predominating 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 transforming amino acids and fats. Osteocyte metabolism is not a well-understood process. Osteoclasts generate energy in the processes of glycolysis and the Krebs cycle (Yang et al., 2020).

Osteoblasts mainly produce the extracellular matrix. Osteocytes contribute to a lesser extent to the production of the matrix, which is due to their structure – mature osteocytes do not have many organelles responsible for secretion (Klein-Nulend and Bonewald, 2020). ECM consists mainly of collagen (90%) and non-collagen proteins (10%). Most collagen is a type I; 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) (Gentili and Cancedda, 2009; Johansen et al., 1992; Lin et al., 2020). Proteoglycans are mainly secreted by osteoblasts, e.g. biglikan (Hua et al., 2020) or decorin (Li et al., 2008). Osteocalcin is primarily secreted by mature osteoblasts but also by osteocytes (Hosseini et al., 2019). SIBLINGs are small hydrophilic proteins containing the characteristic set of amino acids (RGD) and participating in cell adhesion processes. These compounds are mainly produced by osteocytes (e.g. Matrix Extracellular Phosphoglycoprotein (Siggelkow et al., 2004)), but to a lesser extent also by

osteoblasts (osteopontin, Dentin Matrix Protein-1 (Bellahcène et al., 2008; Saito et al., 2020; Singh et al., 2018)). Osteoclasts are also capable of secreting H^+–adenosine triphosphate and cathepsin K protease responsible for dissolving the demineralised organic part of bone during bone resorption processes (Bossard et al., 1996; Teitelbaum, 2000).

Bone markers are examined to determine if the biomaterial supports cell growth. The rate of bone formation is related to the enzymatic activity of osteoblasts, which can be examined by checking the activity of a well-recognised osteogenic marker alkaline phosphate (ALP) (Kara et al., 2019; Sukul et al., 2021). ALP starts to be expressed by osteoblasts when they cease to proliferate, reaches a peak during matrix maturation and eventually, its concentration decreases with the mineralisation process (Garnero and Delmas, 1997; Stein et al., 1990). Similarly, osteopontin (OPN) or osteocalcin (OCN) secreted by mature osteoblasts can be used, reaching its maximum level during mineralisation (Erickson and Payne, 2019; Stein et al., 1989). In vitro studies of MCF-7 cells on chitosan scaffolds showed that chitosan has a positive effect on the metabolic behaviour of cells: the metabolism of cells in such a system is very similar to that in tissue (Dhiman et al., 2005). Similarly, for dental pulp stromal cells, CS has a positive effect on metabolism and proliferation (Amir et al., 2014).

5. CONCLUSIONS

Chitosan (CS) is a polysaccharide exhibiting biocompatibility, biodegradability and suitable physicochemical properties and therefore shows great potential in tissue engineering uses. There has been a great deal of research into the use of CS to produce biocompatible scaffolds that could be used to treat bone damage caused by, for example, ligament reconstruction, tumour resection or osteoporosis. The use of chitosan as one of the scaffold materials alongside others, e.g. polylactide, polycaprolactone, alginate, pectin, hydroxyapatite, improves mechanical and surface properties of the scaffold and shows good cell affinity, promoting cell adhesion and proliferation. However, more focus should be on the interaction between chitosan and the cell and its osteogenic effect. A thorough understanding of these processes will aid the medical treatment of bone damage.

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