SULFONATED AND SULFATED CHITOSAN DERIVATIVES FOR BIOMEDICAL APPLICATIONS: A REVIEW

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Abstract

From 20th century, chitosan, a natural polysaccharide, has received much attention for use in biomedical applications thanks to its remarkable properties, such as biodegradability, biocompatibility, hemostasis and antibacterial activity. Over the last decades, many researchers have attempted to generate new chitosan derivatives-based biomaterials though chemical modifications, especially through sulfonation or sulfation reactions in order to tailor the physicochemical and biochemical properties. Due to the presence of residual amino groups, the generated polyampholytic derivatives are characterized by convenient biological properties, such as antioxidation, antiviral activity, anticoagulation and bone regeneration, expanding their application scope. This paper provides an overview of the strategies used to chemically modify chitosan by introduction of sulfonate groups on chitosan backbone, focusing on various sulfonating or sulfating agents used and substitution regioselectivity, and highlights their applications in biomedical field.

Keywords: chitosan, sulfation, sulfonation, regioselectivity, polyampholyte, biomedical application

Abbreviation list

CS: chitosan, GAG: glycosaminoglycan, DDA: degree of deacetylation, ECM: extracellular matrix, FFSA: 5-formyl-2-furansulfonic acid, BZ1S: 2-formylbenzene sulfonic acid, BZ2S: 4-formyl-1,3-benzenedisulfonic acid, APTT: activated partial thromboplastin time, PT: prothrombin time, TT: thrombin time, MW: molecular weight, LMW: low molecular weight, LPL: lipoprotein lipase, BSA: bovine serum albumin, BFG: bovine serum fibrinogen, LMWH: low molecular weight heparin, HIV-1: human immunodeficiency virus type 1, AIDS: immune deficiency syndrome, MIC: minimum inhibitory concentration, ROS: reactive oxygen species, DDPH: 2,2-diphenyl-1-picrylhydrazyl, BTE: bone tissue engineering, BMP: bone morphogenetic protein, ALP: alkaline phosphatase, hMSC: human mesenchymal stem cell, OB: human primary osteoblast, GCTB: giant cell tumor of bone.

Contents

1. Introduction

Chitosan (CS) is a linear polysaccharide composed of two repeat units, D-glucosamine and N-acetyl-D-glucosamine, linked by β-(1→4)-linkages (Figure 1). It is obtained from the deacetylation of chitin which is the second naturally most abundant polysaccharide that can be extracted from the exoskeleton of crustaceans, squids or fungi walls (Synowiecki & Al-Khateeb, 2003). CS is the only natural positively charged polysaccharide since it possesses a large number of free amino groups ($N\ \%_{-wt}$ = 9.938 % or 6.21 meq. g^{-1} of free amino groups for a CS with degree of deacetylation (DDA) equal to 100 %) making it soluble in neutral or acidic aqueous media (pH <6) depending on the DDA, molecular weight or natural source. These functional groups allow CS to benefit from excellent physicochemical and biological properties such as adsorption, biodegradability, biocompatibility and hemostasis (Franconetti, Contreras-Bernal, Prado-Gotor, & Cabrera-Escribano, 2015; Freier, Koh, Kazazian, & Shoichet, 2005; Islam et al., 2016; M. N. V. R. Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Pereira et al., 2015; Wu, Zhou, Liu, & Wan, 2015; Zhai, Bai, Zhu, Wang, & Luo, 2018). Henceforth, CS has a commercial interest due to its versatile applications; water-waste treatment, air filtration, cosmetic and food industrial, drug delivery, wound healing, etc. However, this natural polysaccharide exhibits a limitation in its reactivity and processability because of a high density of hydrogen bonds between polymer chains in the solid state, and high viscosity due to the presence of intramolecular repulsive electrostatic forces that extend the polymer coil in solution. In literature, researchers have turned to chemical modification of CS to produce new biofunctional materials in order to tailor the raw polymer properties, such as phosphorylation (Shanmugam, Kathiresan, & Nayak, 2016), quaternization (Ren, Zhao, Liang, Ma, & Guo, 2017), carboxyalkylation (Wahid, Wang, Lu, Zhong, & Chu, 2017) and hydroxyalkylation (Shao et al., 2015). The derivatives of CS kept the original properties of chitosan and exhibited as well, new or improved characteristics depending on the nature of additional functions. Xu et al investigated a partially demineralized dentine sections modified by covalent immobilization of phosphorylated CS and showed a better deposition of calcium phosphate on the dentine surface, making it a suitable candidate for bone tissue engineering (Xu, Neoh, Lin, & Kishen, 2011). Likewise, Zhou *et al.* successfully synthesized quaternized CS and highlighted its antibacterial activity against *Staphylococcus aureus* for

use as potential wound dressing for skin regeneration (Y. Zhou et al., 2013). CS chemical modification could occur selectively on the nucleophilic amino groups (C-2 position on amino glucose repeat units), or on the hydroxyl groups (C-3, C-6 positions on both acetyl glucosamine and glucosamine repeat units) or indistinctly on amino groups and hydroxyl groups. Most of researchers have focused on the incorporation of sulfonate groups onto CS via sulfonation reactions. Sulfonate groups could be attached directly on free amino groups producing sulfamate products ($-NH-SO_3^-$) (J. Yang et al., 2015), or introduced via compounds which contain sulfonate groups ($R-SO_3^-$) leading to sulfonated products ($-NH-R-SO_3^-$) (Jung, Na, & Kim, 2007; Lima et al., 2013; Ouerghemmi et al., 2018). The sulfonation reaction may take place on hydroxyl groups resulting into sulfated products ($-O-SO_3^-$) (Gilbert, Veldhuis, Carlson, & Giolito, 1953; Qu, Wu, Yin, & Zhang, 2012) (Figure 1). In this way, the sulfonation reaction may lead to sulfonated and/or sulfated CS derivatives, that will be abbreviated SCS when they cannot be distinguished. Due to the existence of residual amino groups, the resulting SCS chains present polyampholytic characteristics encountered in the structure of some sulfated glycosaminoglycans (GAG), a special class of complex charged polysaccharides involved in the extracellular matrix (ECM) (e.g. chondroitin sulfate, heparin). By interacting with a wide variety of GAG-binding proteins in the ECM (extracellular matrix) and mediating cell signal pathway, GAGs are known to regulate cell behaviors, such as cell adhesion, migration, proliferation and differentiation (Sila et al., 2018; Valcarcel, Novoa-Carballal, Pérez-Martín, Reis, & Vázquez, 2017). In addition, sulfated GAGs have several pharmacological properties and fundamental biological activities, as immunomodulation, antioxidant, antiviral, anti-radiation, anti-inflammatory, neuroprotective, anti-proliferative and anticoagulant effects (Krichen et al., 2017; Pomin, 2015; Sayari et al., 2016; Yue Yu, Shen, Song, & Xie, 2018). Thus, SCS derivatives should benefit from these excellent biological properties depending, among others, on the degree of substitution. Indeed, SCS derivatives showed attractive properties, for example antimicrobial (Sun, Shi, Wang, Fang, & Huang, 2017), antioxidant (Seedevi, Moovendhan, Vairamani, & Shanmugam, 2017), water solubility (Tang et al., 2011), etc. Furthermore, SCS have been employed for their blood anticoagulant properties ascribable to their similar chemical structure with that of heparin (Clayton Souza Campelo et al., 2016; Shih-Hang Chang & Huang, 2012). Moreover, sulfated CS have been used as delivery systems for tissue repair and regeneration thanks to their capacity for binding to protein growth factors, and showed to be the most efficient sulfated derivatives to direct neural differentiation (Doncel-Pérez et al., 2018). Therefore, SCS derivatives are very promising candidates for a wide range of applications such as drug delivery (Sandhya et al., 2018), bone tissue engineering (Kong, Wang, Cao, Yu, & Liu, 2014), blood contact devices (Campelo, Chevallier, Vaz, Vieira, & Mantovani, 2017), etc.

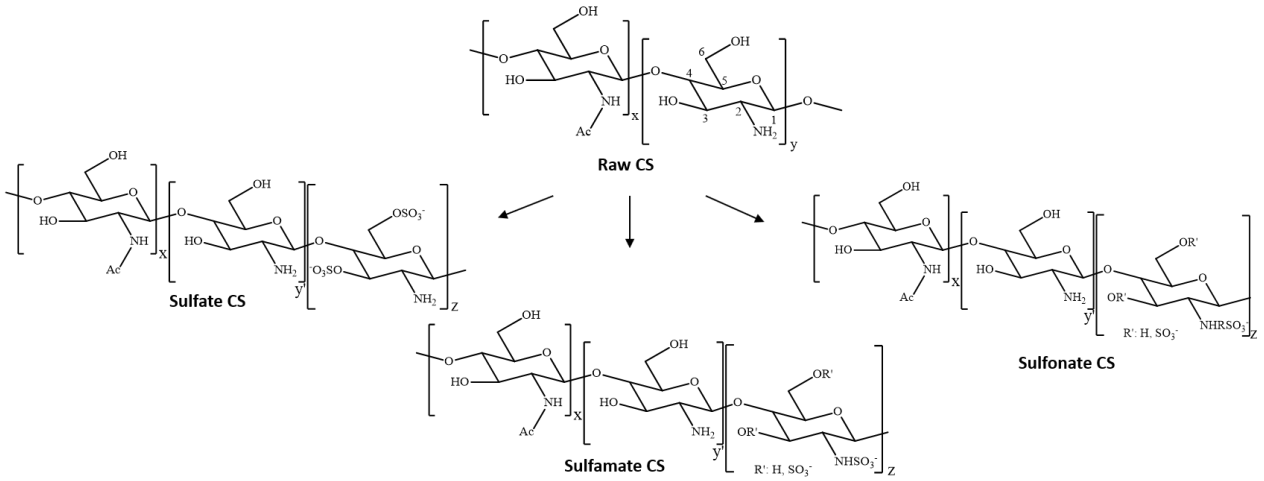

Figure 1. Chemical structure of raw chitosan and its sulfonate, sulfamate and sulfate derivatives

The sulfonation of chitosan to improve its biological properties has been considered in several publications (Figure 2). However, a few review articles analyzing recent studies on the synthesis and the potential applications of SCS derivatives have been reported (Alves & Mano, 2008; Jayakumar, Nwe, Tokura, & Tamura, 2007). Jayakumar *et al.* investigated the synthesis of sulfated chitin and chitosan, without intending the difference between sulfonation position, and highlighted few applications (Jayakumar et al., 2007). Likewise, Alves and Mano described the different ways to chemically modify both chitin and chitosan, as graft copolymerization, the eventual combination with cyclodextrins and reported their biomedical and environmental applications (Alves & Mano, 2008). Besides, these review papers specifically focused on SCS have been submitted before 2009 and as shown in Figure 2, there is an increase in number of publications concerning the sulfonation or sulfation of chitosan since 2009. This review paper firstly intends to present an updated state of the art on the different strategies to synthesize SCS derivatives, focusing on the regioselectivity as selective O-, or N-substitutions, and the effect of chemical modification on physicochemical and biological properties. Our second goal is to highlight the new trends of SCS in biomedical applications, such as bone tissue engineering and antioxidant applications, and subsequently to come up to perspectives.

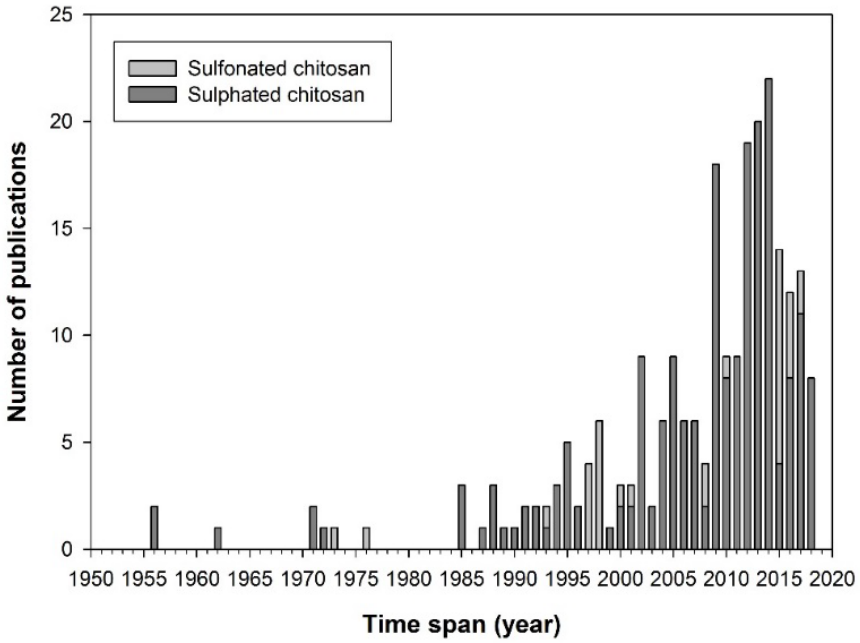

Figure 2. Number of publications related to sulfonated chitosan and sulfated chitosan derivatives, source: SciFinder, keywords: sulfonated chitosan, sulfated chitosan, retrieved on 19 March 2018

2. Strategies to Synthesize Sulfonated and Sulfated Chitosan

Over the years, a great number of studies on the chemical modification of CS has been reported (Alves & Mano, 2008; Bedini, Laezza, Parrilli, & Iadonisi, 2017; Shukla, Mishra, Arotiba, & Mamba, 2013; Sobahi, Abdelaal, & Makki, 2014). The synthesis of SCS derivatives has been well studied in order to improve biological properties of raw CS such as anticoagulant activity (Balan & Verestiuc, 2014; Campelo et al., 2016; Jayakumar et al., 2007; T. Wang et al., 2012). Even if the amino groups are known to be more nucleophilic compared to hydroxyl groups, the chemical substitution could occur at three key positions in the glucosamine and acetyl glucosamine residues: C-2, C-3 and C-6 positions carrying amino, secondary and primary hydroxyl groups, respectively (Ahmed, Annu, Ali, & Sheikh, 2018; Vongchan, Sajomsang, Subyen, & Kongtawelert, 2002). This gives rise to N-modified, O-modified or N,O-substituted CS by one-step or multiple-steps reactions (Figure 3). Depending on the type of sulfating reagent and reaction conditions, the reaction could lead to a selective or non-selective derivative (Verlee, Mincke, & Stevens, 2017). Both amino and hydroxyl groups could react with electrophilic agents such as alkyl halides, acids or iso(thio) cyanides leading to the formation of N- and O-modified CS derivatives.

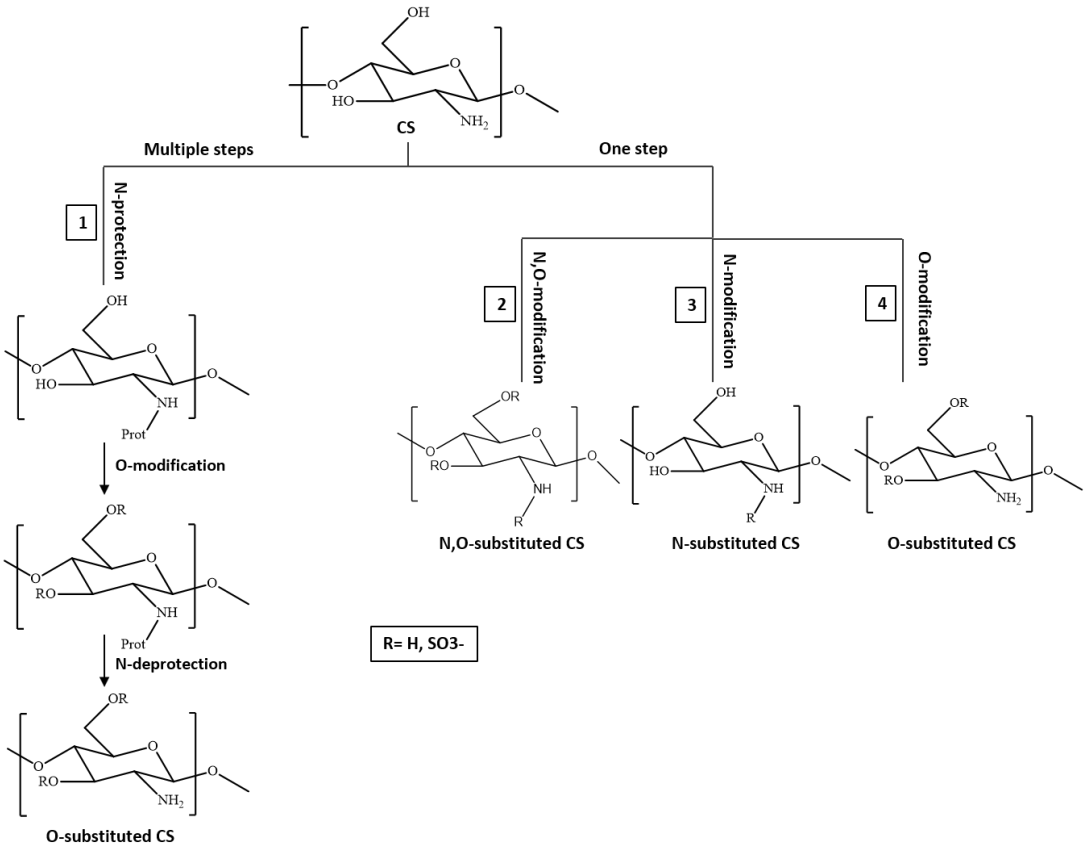

Figure 3. Overall strategies used to chemically modify chitosan. 1) selective substitution in O-position after N-protection/ deprotection, 2) non-selective substitution in N- and O-positions, 3) selective substitution in N-position and 4) selective substitution in O-position without N-protection/ deprotection

Several combinations of sulfating or sulfonating reagents and reaction media have been investigated for the sulfonation of polysaccharides (Figure 4). The insolubility or low solubility of polysaccharides in organic solvents used as reaction media leads to heterogeneous products since the reaction is conducted under heterogeneous or semi-heterogeneous conditions. In addition, harsh conditions can lead to severe molecular degradation by breakage of the glucosidic linkages of the polysaccharide, other side reactions, poor reproducibility (Shigehiro Hirano, Tanaka, Hasegawa, Tobetto, & Nishioka, 1985; R. Huang, Du, & Yang, 2003; R. Huang, Du, Yang, & Fan, 2003; Nagasawa, Tohira, Inoue, & Tanoura, 1971; Nishi et al., 1986). In the last two decades, researchers have tried to overcome these drawbacks (Gamzazade et al., 1997; Nishimura et al., 1998; K. Zhang et al., 2010).

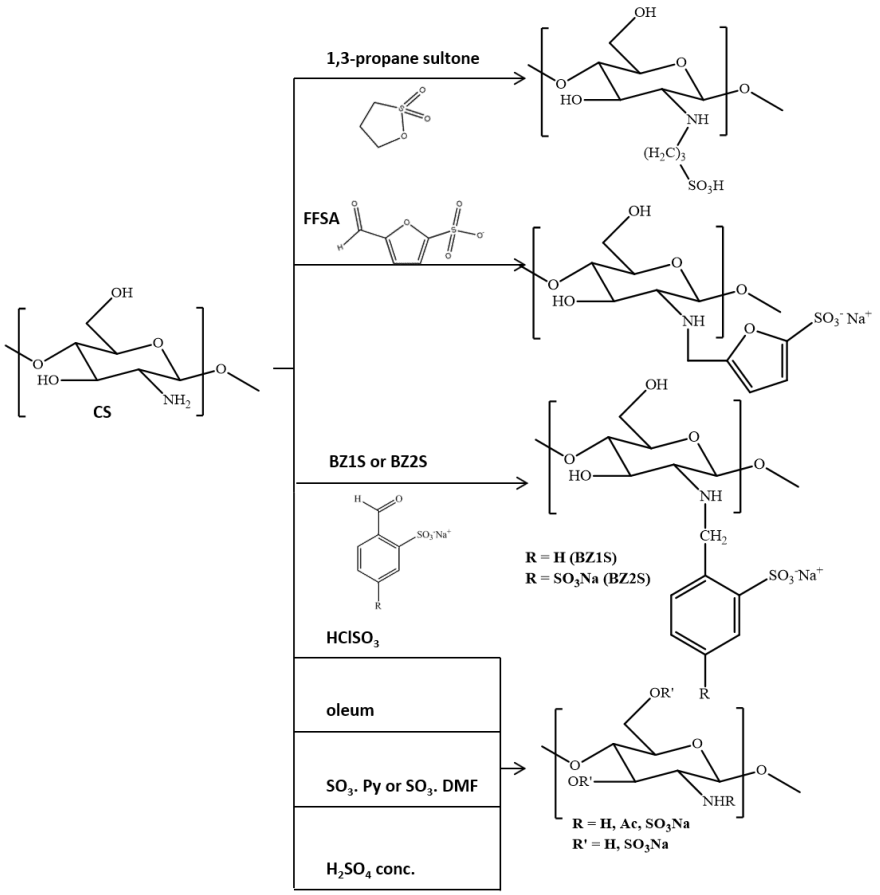

Figure 4. Several sulfonate reagents mostly used to synthesize N,O- substituted chitosans.
FFSA: 5-formyl-2-furansulfonic acid sodium salt, BZ1S: 2-formyl benzene sulfonic acid sodium salt, BZ2S: 4-formyl-1,3-benzene disulfonic acid disodium salts

These experimental conditions of chemical modification (sulfating reagents, solvents, temperature and reaction time, etc.) could have effect on various CS derivative parameters i.e. molecular weight (MW), DS, solubility, etc. (Figure 5). For instance, even when employed under controlled conditions, sulfuric acid could cause extensive CS chain degradation, as could do oleum, chlorosulfonic acid and sulfur trioxide when applied alone, which could explain the lack of information about MW variation in several research articles. However, these two reagents were widely used when combined with Lewis bases because they caused less degradation (Vongchan, Sajomsang, Kasinrerk, Subyen, & Kongtawelert, 2003). Additionally, depending on the sulfonating reagent and reaction media, the sulfonation synthesis could occur through different systems: heterogeneous, homogeneous and pseudo-homogeneous resulting in different SCS derivatives (Gamzazade et al., 1997; K. Zhang et al., 2010). As a matter of fact, different SCS derivatives could be obtained after sulfonation reaction from the same original CS. Basically, SCS derivatives possess similar chemical composition but differ each other by structure as this may be related to the regioselectivity which results from various reactivities of the three functional groups of the parent polymer. Consequently, mono-SCS derivatives, di-SCS compounds or copolymers containing both monosulfonated and disulfonated blocs randomly or homogeneously distributed on polymer chain could be obtained.

In conclusion, the important factors mentioned above and the origin of CS should be taken into account as they influence the physicochemical properties of the obtained products (Ahmed et al., 2018). Besides, there is a relationship between the physicochemical properties and biological properties. Thus, biomedical applications of SCS derivatives depend on the chemical modification process (see next section "Properties and Biomedical applications of sulfonated and sulfated chitosan").

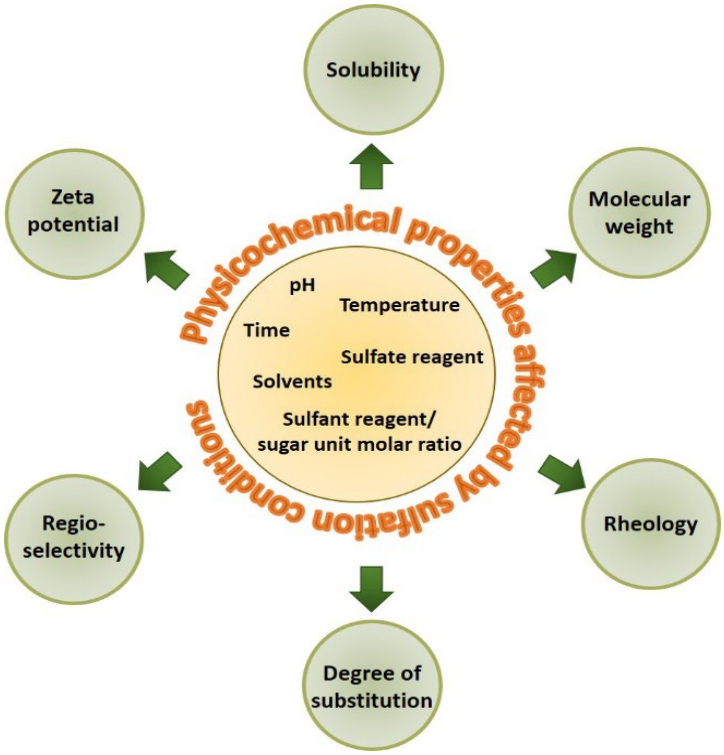

Figure 5. A scheme of properties affected by the sulfonation modification of CS

The strategies applied to synthesize SCS derivatives can be classified according to substitution position.

2.1. N,O-substitution

Various methods that involve N,O-substitution have been used for the sulfonation of polysaccharides (Ghaee, Nourmohammadi, & Danesh, 2017; S. Hirano, Hasegawa, & Kinugawa, 1991; Shigehiro Hirano et al., 1985; R. Huang, Du, Yang, et al., 2003; Vongchan et al., 2002; Whistler & Kosik, 1971; Xing et al., 2004; H. Zhou et al., 2009). N-succinyl CS sulfates were prepared by reacting N-succinyl CS with sulfating agent $N(SO_3Na)_3$, previously synthesized, in aqueous solution (T. Wang et al., 2012). The FT-IR and NMR spectra confirmed that sulfonate introduction reaction took place on primary and secondary hydroxyl groups and also on amino groups, and the highest DS (1.97) were obtained under optimal conditions at 50°C for 20h. Furthermore, they evidenced that the DS was dependent on sulfating agent/ N-succinyl CS ratio, reaction temperature, reaction time and pH of sulfating agent. DS increased (from 0.01 to 0.74) with the increase of pH (from 3 to 9) but deceased upon further increasing pH (> 9). By increasing the ratio sulfating reagent/ sugar unit (from 0.75/283 to 3/283) and sulfating time (from 5h to 20h), DS increased and became

constant with prolonged sulfating time and at higher ratio due to steric effects and electrostatic repulsion. Besides, DS rose with the temperature (from 20°C to 50°C) then decreased with further increase of temperature. This phenomenon is due, on one hand, to the destruction of crystalline regions of N-succinyl CS that became amorphous and to the formation of active reaction centers that accelerated the sulfation reaction by increasing the temperature. On the other hand, the high temperature hampered the reaction and made the chemical equilibrium move to the opposite direction since the sulfation is an exothermic reaction (T. Wang et al., 2012). These observations are in agreement with those obtained by Yang *et al.* (J. Yang et al., 2013). They prepared N,O-sulfated CS by using trimethylsilylated CS and sulfur trioxide – pyridine (SO_3 - Py) complex in anhydrous DMSO (J. Yang et al., 2013). Silylation reaction occurred before sulfating modification in order to improve CS solubility in organic solvents as sulfation reaction took place under homogeneous conditions resulting in higher DS. In their work, different MW of CS were selected and characterizations were investigated in order to study the effect of sulfation conditions on MW and DS. A significant decrease of MW was noticed indicating chains scissions reactions. However, during sulfation from trimethylsilylated CS, the chain scission was decelerated and by increasing the degree of trimethylsilylation, the reduction of MW was less marked. The depolymerization of sulfated polysaccharide could be related to the presence of free hydroxyl groups on molecular chains during sulfation (J. Yang et al., 2013). Moreover, harsh sulfation conditions could reduce MW of the sulfated products: the increase in sulfating reagent to sugar unit molar ratio, reaction time and temperature lead to a gradual decrease of MW of final products. Thus, under optimal conditions, Yang *et al.* prepared highly sulfated CS powder with high (DS>2) under homogeneous condition and showed that the resulting anticoagulant activity was strongly dependent on the concentration and DS (J. Yang et al., 2013). N,O-Sulfated CS was also synthesized by treating CS with oleum (Gamzazade et al., 1997) in formamide – Dichloroacetic acid and a DS of about 1.10-1.63 was obtained under semi-heterogeneous conditions (Vikhoreva et al., 2005).

Interestingly, sulfated CS derivatives were synthetized by Xing *et al.* under microwaves yielding reaction products with a high DS and low MW with the use of DMF. SO_3^- as sulfating reagent and DMF – formic acid as reaction media in a shorter time (Xing et al., 2004). These authors proved that reaction under microwave radiations is a convenient method to obtain a wide range of sulfated CS derivatives with different DS and MW only by changing reaction time or/ and radiation power. Besides, no differences in chemical structures were observed between microwave and traditional technologies. Nevertheless, the degradation of sulfated CS seemed to be accelerated with microwave radiations, and the MW was considerably lower than that obtained by conventional methods (Xing et al., 2004).

2.2. N-substitution

Several modifications have been performed on polysaccharide's amino groups in order to make novel CS derivatives with interesting physicochemical and biological properties. Rwei and Lien (Rwei & Lien, 2013) have reported the preparation of N-sulfopropyl CS derivative through a ring-opening reaction by using 1,3-propane sultone as a sulfonating reagent and aqueous acetic acid solution (2 wt%) as solvent, under nitrogen atmosphere and at 30°C. Even if it is known to be a potent carcinogen (Bolt & Golka, 2004), 1,3-propane sultone has been widely used to form sulfopropyl CS derivatives (Jung et al., 2007; Tsai, Wang, Lin, & Lien, 2010). Thus, in order to remove the unreacted sultone, the reaction should be complete and followed by additional purification steps which make the preparation of

sulfonated CS crucial and costly (Rwei, Chen, Lin, & Chiang, 2014). The resulting sulfonated CS showed a water-soluble property. Rwei and Lien carried out the rheological characterizations and found that the rheological properties of sulfonated CS could be affected as the following factors are increased in the order of declining effect; pH level > temperature > salt concentration (Rwei & Lien, 2013). With increasing pH, the slope of loss modulus G" and the quasi-plateau region of store modulus G' decreased, as the protonation effect would be suppressed when pH rose in the sulfonated CS aqueous solution making the water become a poor solvent similarly to what happens with raw CS. In addition, at a low pH environment (pH 2) and for a 5 wt% sulfonated CS solution, the effect of adding various salt concentrations, as sodium chloride, was negligible. In fact, Na^+ or Cl^- did not inhibit the interaction between the sulfonic acid group and the protonated ammonium group, so the conformation of the sulfonated CS did not shrink nor swelled with the addition of various amounts of NaCl at a low pH. Likewise, the dynamic viscosity of sulfonated CS was insensitive to the temperature variation as the change of temperature (from 22 to 50 °C) had an insignificant effect on G". Nonetheless, with increasing temperature, the quasi-plateau of G' at low frequency became more pronounced, since the hydrogen bonding between the water molecules and sulfonated CS were weakened and the water molecules were expelled by the sulfonated CS network (Rwei & Lien, 2013). N-sulfofurfuryl CS derivatives, carrying anticoagulant activity, have also been synthesized, under mild conditions of the Schiff reaction, by the use of 5-formyl-2-furansulfonic acid, sodium salt (FFSA) in methanol or aqueous solution in order to avoid polymer degradation and O-substitution (Amiji, 1998; Campelo et al., 2017, 2016; Lima et al., 2013; Liu, Zhang, Cheng, Cao, & Yao, 2004; Muzzarelli, 1992). Muzzarelli prepared N-sulfofurfuryl CS, with a degree of substitution (DS) of 0.26 ± 0.02 and a content of free amino groups was about 0.32 ± 2 (Muzzarelli, 1992). The same reaction has been reported by Amiji (Amiji, 1998) who demonstrated that, unlike CS which is soluble only in acidic medium, the obtained N-sulfofurfuryl CS derivative is soluble over a wide range of pH (from pH 2 up to pH 12) , highlighting its amphoteric character. The elemental analysis confirmed that the sulfur content was about 5.20% and the DS 23.4%. The platelet adhesion and activation evidenced the non-thrombogenic properties of this N-substituted CS derivative and its suitable use for some blood-contacting applications. Lima *et al.* described the N-sulfonation of CS films, previously shaped by solvent evaporation method, via reductive amination reaction by using FFSA as sulfonating reagent and sodium borohydride ($NaBH_4$) as reductive agent. The CS films were initially transparent, homogeneous with smooth surface. After sulfonation reaction, the surface morphology appeared rougher and a yellowish color was observed, probably due to remaining imine bonds that were not reduced by $NaBH_4$. The introduction of sulfonic acid groups into CS could also occur via a one-step reductive amination by treating CS with 2-formylbenzenesulfonic acid, sodium salt dihydrate (BZ1S) or 4-formyl-1,3-benzenedisulfonic acid, disodium salt hydrate (BZ2S) in order to prepare N-monosulfonated CS and N-disulfonated CS derivatives, respectively (Crini, Gimbert, et al., 2008; Crini, Martel, & Torri, 2008; Crini et al., 1997; Weltrowski, Martel, & Morcellet, 1996). Crini *et al.* reported that disulfonate derivative was effective for the removal of cationic Basic Blue 9 (BB9) (Crini, Martel, et al., 2008) and Basic Blue 3 (BB3) (Crini, Gimbert, et al., 2008) dyes from waste water. They proved that the adsorption capacity depended on the presence and the position of SO_3^- groups on the aromatic cycle, due to the formation of electrostatic interaction between sulfonate groups of CS and cationic dye. Similarly, Martel *et al.* functionalized chitosan coated textile filters with either BZ2S and BZ1S in order to transform them into strong cation exchangers and evidenced their sorption ability of lead and chromium ions in acidic solutions (Martel, Weltrowski, Morcellet, & Scheubel, 1995). Recently, Martel's team published an extensive

study of the synthesis of the afore mentioned polyampholytic aryl- mono and di-sulfonated CS derivatives and defined the optimal conditions of reductive amination reaction to reach *in vitro* anticoagulant activity by varying molar ratios (R) of both sulfonic aldehydes BZ1S and BZ2S *versus* CS free amino groups (Ouerghemmi et al., 2018). The zeta potential (ZP) measurements displayed that all sulfonated CS derivatives, initially solubilized in 0.01 M NaOH, exhibited strongly negative ZP at physiological pH (between -19 and -47 mV) in contrast with parent CS, which displayed global neutral electric charge around pH 8. For all sulfonated CS, the ZP progressively rose with the decrease of pH (from 11 to 4), as the residual glucosamine residues in polymers underwent gradual protonation upon HCl addition. Besides, the study highlighted that both N-arylmonosulfonated and N-aryldisulfonated CS exhibited anticoagulant activities that were concentration and DS dependent. Shelma and Sharma prepared submicroparticles of lauroyl sulfated CS (LSCS), by using sulfobenzoïc acid cyclic anhydride in methanol, which can be used for enhancing hemocompatibility of CS. Indeed, these derivatives showed a significant effect on hemolysis, erythrocytes, leucocytes, platelet aggregation, C3 protein depletion assay and whole blood clotting time, compare to the native CS, expanding their applications in the medical field (Shelma & Sharma, 2011).

2.3. O-substitution

Over several decades, the chemical modifications on hydroxyl groups present in skeleton of CS chains have been reported. The combination of chlorosulfonic acid ($HClSO_3$) as sulfating reagent and dimethylformamide (DMF) as reaction medium has been widely used to add sulfonate functions on hydroxyl groups of CS (Han, Zeng, Zhang, Zhang, & Zhang, 2016; Qu, Yao, Zhang, Wu, & Ping, 2009; Xiang et al., 2010; C. Zhang, Ping, Zhang, & Shen, 2003a; C. Zhang, Qineng, & Zhang, 2004; C. Zhang, Qu, Sun, Wu, et al., 2008; K. Zhang et al., 2010). Han *et al.* studied different substitution positions and prepared 6-O-sulfated CS by using $HClSO_3$/ H_2SO_4 system, 3,6-O-disulfated CS by $HClSO_3$/ DMF combination and 3-O-sulfated CS by 6-O-desulfation of 3,6-O-disulfated CS in order to develop a reliable HPLC separation and UV detection-based assay that could be used to perform routine monosaccharide composition analysis for sulfated CS for quality control purpose (Han et al., 2016). Similarly, N-octyl-O-sulfate CS derivative was prepared and showed potential application as drug delivery system for chemotherapy (Qu et al., 2009). Besides, Terbojevich *et al.* selectively synthesized 6-O-sulfate through two methods (Terbojevich et al., 1989), the first one consisting in using a 2:1 mixture of sulfuric and chlorosulfonic acids and the second one involved the use of a pyridine-SO_3 complex, after protection of the amino and C3-OH groups with copper ions. On contrary to product obtained from method 1 afore mentioned, a depolymerization and a molecular and structural heterogeneity have been observed on the C6-O-sulfate CS prepared by method 2 probably due to the use of nitrous acid and the presence of ordered structures in highly acetylated regions of the parent CS, respectively. Zhang *et al.* investigated the regioselective synthesis of sulfated CS with chlorosulfonic acid in DMF in homogeneous and non-homogeneous conditions (K. Zhang et al., 2010). In order to prepare O-sulfated CS in homogeneous conditions, CS was dissolved in formic acid and $HClSO_3$ in DMF was added slowly. However, for non-homogeneous sulfation, CS was activated firstly with 4% sodium hydrogen carbonate, then $HClSO_3$ in DMF was added. Indeed, a previous dissolution or activation was found to be essential for the sulfation, which seemed to be a dominant substitution of primary hydroxyl groups. Moreover, the total and partial DS (between 1.13 and 1.67 for homogeneous sulfation and between 0.86 and 1.67 for non-homogeneous substitution

could be regulated by varying the reaction conditions. In fact, DS decreased at a high temperature (> 50°C) probably due to the desulfuration and degradation reactions. A lower total and partial DS was obtained by the application of a larger volume of formic acid and the prolongation of dissolving duration (K. Zhang et al., 2010).

Furthermore, it is possible to selectively synthesize O-substituted CS by the use of acidic solvents such as methanesulfonic acid, that make the amino groups protonated and protected (Nishi et al., 1986; Sashiwa, Kawasaki, Nakayama, Muraki, Yamamoto, & Aiba, 2002; Sashiwa, Kawasaki, Nakayama, Muraki, Yamamoto, Zhu, et al., 2002). Badawy *et al.* used this strategy to prepare N,O-acyl CS derivatives and showed that the protection of the amino group by the salt formation with $MeSO_3H$ was nearly quantitative (90.6-100%) (Badawy et al., 2004). Nevertheless, the use of such acids affects the biological activity of CS due to its partial depolymerization. To overcome these mismatches, a protection-deprotection of amino groups method is chosen (Kurita, Ikeda, Shimojoh, & Yang, 2007; Kurita, Ikeda, Yoshida, Shimojoh, & Harata, 2002; C. Zhang, Ping, Zhang, & Shen, 2003b). Yeh and Lin attempted the introduction of sulfonate/ sulfonic groups onto the C6 hydroxyl groups of raw CS in order to carry out a surface modification of CS film (Yeh & Lin, 2008). They protected the amino function by introducing N-ethoxycarbonylphthalimide to avoid its reaction with the sulfonating agent and confirmed that after deprotection step, film surface CS amino groups could be partially recovered. The amount of recovered amino groups enhanced with the increase of concentration of the deprotective reagent, hydrazine monohydrate. However, even at very high concentration, there were still some protected groups left and cleavage of grafted sulfonate/ sulfonic functions and either linkage along the CS backbone could occur (Yeh & Lin, 2008). Otherwise, the zeta potential measurements evidenced that the surface charge of raw CS film has reached neutrality at physiological condition (i.e. pH 7.4). Conversely to direct sulfated CS membrane that showed negative surface zeta potential due to the high number of sulfonate/ sulfonic groups, sulfated CS films, obtained after protection – deprotection strategy, remained positive in lower pH region. This result could be attributed to the presence of high number of amino groups recovered after deprotection.

Table 1 summarizes effects of several sulfonation conditions on physicochemical properties of CS derivatives.

317 **Table 1.** Effects of some factors on physicochemical properties of sulfonated/ sulfated CS derivatives (DS = degree
318 of substitution ; MW = molecular weight ; ZP = zeta potential)

	DS	MW	Rheology	ZP
pH	DS increases with the increase of pH from 3 to 9 DS decreases when high pH (>9) (T. Wang et al., 2012)		G" and G' decrease with the increase of pH (Rwei & Lien, 2013)	ZP increases with the decrease of pH (Ouerghemmi et al., 2018)
Sulfating or sulfonating reagent to sugar unit molar ratio	DS increases with the increase of molar ratio and became constant at higher ratios (Ouerghemmi et al., 2018; T. Wang et al., 2012; J. Yang et al., 2013)	MW increases with the decrease of molar ratio (J. Yang et al., 2013)		ZP decreases with increasing molar ratio (S. Ouerghemmi et al., 2018)
Time	DS increases with prolongation of reaction time (T. Wang et al., 2012; J. Yang et al., 2013; K. Zhang et al., 2010)	MW decreases with prolongation of reaction time (J. Yang et al., 2013)		
Temperature	DS increases with the increase of temperature DS decreases at high temperature (>50°C) (T. Wang et al., 2012; J. Yang et al., 2013; K. Zhang et al., 2010)	MW decreases with increase of temperature (J. Yang et al., 2013)	G" insensitive to the temperature change G' increases with the increase of temperature (20 - 50°C) (Rwei & Lien, 2013)	
Salt concentrations			Negligible effects on G" and G' at low pH (pH 2) (Rwei & Lien, 2013)	

319 **3. Properties and Biomedical Applications of Sulfonated and Sulfated Chitosan**

320 Besides environmental applications based on the sorption of cationic metallic or organic species by ion exchange
321 mechanism with strong acidic groups on CS backbone, (Abu-Saied et al., 2017; Crini, Gimbert, et al., 2008; Y. Huang
322 et al., 2018; Lv, Liu, Zhou, Huang, & Sun, 2014; Shirdast, Sharif, & Abdollahi, 2016; Weltrowski et al., 1996), SCS
323 derivatives are widely used in biomedical field thanks to their polyampholytic character and to their specific
324 physicochemical properties.

3.1. Hemocompatible biomaterials

Recently, various biomaterials that imply a blood contact, made of synthetic (poly (caprolactone), poly (lactic acid), poly (tetrafluoroethylene)) or natural (cellulose, alginates, chitosan) polymers have been developed in biomedical areas, such as artificial organs, heart valves, vascular prostheses, etc (Balan & Verestiuc, 2014; Coimbra et al., 2017; Gravastrand et al., 2017; M.-K. Lee, Park, Jang, Kim, & Jeong, 2018; Muxika, Etxabide, Uranga, Guerrero, & de la Caba, 2017; Santoro, Shah, Walker, & Mikos, 2016). Despite the promising results obtained from synthetic biomaterials, the lack of surface hemocompatibility remains one of the main drawbacks (Ratner, 2007). This property is substantially managed by blood materials interactions, as platelet and protein adsorptions on biomaterials, and by coagulation biochemistry (Balan & Verestiuc, 2014). Indeed, biomaterial–blood interactions could be explained by the adsorption of plasma proteins, especially clotting enzymes and fibrinogen, followed by the adhesion and activation of platelets inducing thrombus' formation (Othman, Cillero Pastor, van Rijt, & Habibovic, 2018). The formation of thrombus, that can lead to serious health problems, such as cerebral thrombosis, pulmonary embolism, coronary artery clots and stroke, could be prevented by the use of anticoagulants. Heparin, a naturally occurring GAG, is the most widely used and effective blood anticoagulant drug administered to patients for treatment or prevention of thrombosis. However, recent studies evidenced that heparin has adverse effects limiting its use in several clinical cases, for example easy blending and bruising, feet itches, bluish-colored skin, pain, redness, warmth or skin changes where the medicine was injected, and serious side effects as thrombocytopenia and thrombosis syndrome (Roberts & Chaney, 2018). Therefore, people have turned to developing novel alternatives to heparin. Heparin presents a complex polysaccharidic structure mainly composed of D-glucuronate sulfate and N-sulfoglucosamine-6-sulfate, therefore CS sulfation is a way of choice to reach products of close composition and subsequently similar physicochemical and biological properties.

As a matter of fact, various studies reported that the resulting SCS derivatives possess blood anticoagulation activity as they displayed ability to delay the clot formation (Balan & Verestiuc, 2014; Baoquan, Weilin, Wei, & Junkai, 2007; R. Huang, Du, Yang, et al., 2003; Ramasamy et al., 2017; Suwan et al., 2009; J. Yang et al., 2013). Blood coagulation is a complex process, which can be broadly divided into three stages, namely the formation of prothrombin activator, the conversion of prothrombin to thrombin and conversion of fibrinogen to fibrin. It has been demonstrated that the anticoagulant activity was, most of time, lower than that of heparin (Drozd et al., 2001; T. Wang et al., 2012). Besides, an anti-thrombogenic character (Amiji, 1998) and no or low platelet adhesion and activation, which could be explained by the decrease in free amino groups since some of them were used in the sulfonation modification (Campelo et al., 2017; Yeh & Lin, 2008), were evidenced. Indeed, amino groups are responsible for the platelet adhesion and thus for the hemostatic property of CS (Okamoto et al., 2003). In order to investigate blood coagulation, workers mostly evaluate the effect of biomaterial on the activation of clotting factor cascade. Activated Partial Thromboplastin Time (APTT) evaluates the intrinsic and common pathway, Prothrombin Time (PT) assesses the extrinsic and common pathway and Thrombin Time (TT) is used to study the thrombin activity of fibrin polymerization. According to the published literature, SCS derivatives could effectively prolong APTT even at low concentration, and depending on the type of substituted sulfonated group, slightly or not prolong TT. Nevertheless, no or weak effect is observed on PT. In fact, heparin and heparinoids generally interfere with the last step of the intrinsic coagulation pathway and show no or

slight effect on the extrinsic pathway. It has been reported that the anticoagulation activity was closely related to MW, DS, and concentration of sulfonated CS (R. Huang, Du, Yang, et al., 2003; Maruyama, Toida, Imanari, Yu, & Linhardt, 1998; Muzzarelli et al., 1984; Nardella et al., 1996; Vikhoreva et al., 2005). The relationship between MW and anticoagulant activity has been examined and the results were very controversial. Some studies showed that APTT increased with the increase of MW and decreased when it reached a specific value (2×10^4 g.mol^{-1}) (T. Wang et al., 2012), while others noticed that the highest fractions of MW CS polysulfate resulted in a higher anticoagulant activity using TT assay and that the lowest MW fractions exhibited an increase in anticoagulant activity of APTT (Shigehiro Hirano et al., 1985; R. Huang, Du, Yang, et al., 2003; Suwan et al., 2009; Vongchan et al., 2002). Thus, there is no clear relationship between MW and anticoagulant activity (J. Yang et al., 2013). Moreover, several studies revealed that, compared to MW, DS and concentration were obviously more important parameters to consider, as the clotting time was significantly prolonged with the increase of both DS and concentration (Ouerghemmi et al., 2018; T. Wang et al., 2012; J. Yang et al., 2013).

These hemocompatible sulfated/ sulfonated biomaterials have been exploited by applying different strategies and under various shapes of materials i.e. membranes (Lima et al., 2013; Xue, Zhao, Nie, Sun, & Zhao, 2013; Yeh & Lin, 2008), film coating on TiNi alloy implant (Chang & Huang, 2012), covalent grafting on medical devices in contact with blood via functionalization with polydopamine (Campelo et al., 2017), submicroparticles (Shelma & Sharma, 2011).

In the case of the development of biomaterials for blood contact, such as heart valves, calcification phenomena are identified as a clinical concern frequently encountered (Aimoli, Torres, & Beppu, 2006; Park et al., 1997). Literature reports that sulfonated and sulfated groups provide anticalcifying activity to biomaterials surfaces and displays the most probable mechanism occurring (Campelo et al., 2016; W. K. Lee et al., 2000; Park et al., 1997). First, negatively charged sulfonated groups are thought to induce the rapid formation of calcium phosphate compounds by electrostatic interactions with calcium ions. However, over a second phase, the strong acidic sulfonate groups may cause a local decrease of pH that would increase solubilization of calcium-based compounds preventing thereby the formation of calcifying nuclei during the early stage of calcification. In this way, Campelo *et al.* reported the anticoagulant and anticalcifying activities of CS film modified with 5-formyl-2-furansulfonic acid as sulfonate agent (Campelo et al., 2016). The effect of SCS derivatives on apatite formation is very controversial since several research studies showed their potential use in bone tissue engineering field. This will be discussed in the next sub-section.

In addition to anticoagulant and anticalcifying activities, sulfate CS derivatives benefit from other biological properties such as lipoprotein lipase (LPL) release activity. O-sulfated N-acetylchitosan showed a two times higher anticoagulant activity and a 10% LPL-releasing activity than those of heparin (S. Hirano & Kinugawa, 1986). Besides, research studies have demonstrated that sulfated derivatives of CS, prepared from three kinds of partially deacetylated CS (90%, 75% and 50%) could improve solubility and prolyl endopeptidase (PEP) inhibitory activities (Je, Park, & Kim, 2005). 50%-deacetylated CS sulfate exhibited the best inhibitory activity and inhibition rate was dose-dependent. Xue *et al.* reported the synthesis of a macromolecular additive made of sulfated CS derivative blended with

polyethersulfone (PES) for the elaboration of modified PES membranes that presented improved blood compatibility compared to pristine PES membrane (Xue et al., 2013). These blended membranes showed an improved hydrophilicity and lower bovine serum albumin (BSA) and bovine serum fibrinogen (BFG) adsorption, leading to suppressed platelets adhesion and a prolongation of clotting time. These results indicated that these modified membranes could be used in blood purification including hemodialysis.

3.2. Bone tissue engineering

Bone is a natural, nanoscale composite that contains both organic compounds, such as collagen fibrils and mineral components, mainly composed of hydroxyapatite. It acts as smart material since it is capable of regeneration and wound healing by itself. Nevertheless, these natural healing could be insufficient in case of bone defects such as abnormalities, infection, trauma or large bone defects, which represent major challenges in clinical surgery and bone repair materials. Bone grafts is the second most common transplanted tissue (W. Wang & Yeung, 2017). Over than 500,000 bone grafting procedures are counted annually in the United States and 2.2 million worldwide in order to repair bone defects in orthopedics, neurosurgery and dentistry (Pountos & Giannoudis, 2016). Bone tissue engineering (BTE) has turned to the design of new functional materials as bone substitutes that mimic the structure and properties of ECM of native bone, aiming the repair or replacement of bone defects and the improve of biological function of damaged bone (Amini, Laurencin, & Nukavarapu, 2012; Shrivats, McDermott, & Hollinger, 2014). To successfully fabricate such biosubstitutes, three main components are required: isolated cells, tunable factors and supporting scaffolds (Preethi Soundarya, Sanjay, Haritha Menon, Dhivya, & Selvamurugan, 2018; Turnbull et al., 2018). A great interest has been received to biodegradable polymeric scaffolds, such as CS. Thanks to its hydrophilic surface, CS can promote cell adhesion and proliferation, making it ~~an~~ attractive as bone scaffold material. Many studies reported that the presence of sulfate or sulfonic groups in the polymer structure induced nucleation of apatite in a body environment (Leonor et al., 2007) and immobilization of growth factors (Kim et al., 2014). It is worthy to notice that sulfated polysaccharides enhanced the biological activity of bone morphogenetic proteins (BMP), that are growth factors implicated in bone formation and bone tissue reconstruction at an ectopic site (Takada et al., 2003). Furthermore, heparin, a sulfated polysaccharide, prolonged the half-life of BMP-2 by nearly 20-fold and improved BMP-induced osteoblast differentiation *in vitro* and *in vivo* by protecting BMPs from degradation and inhibition by BMP antagonists (B. Zhao et al., 2006). Thereby, many works have been done using sulfonated CS which has close chemical composition to that of heparin as mentioned above, to make new biomaterials suitable for BTE. The release profile of BMP-2 increased with the addition of sulfated CS to calcium-deficit hydroxyapatite (CDH) loaded with BMP-2 (J. Zhao et al., 2011). The effect of sulfate group position, MW and sulfur content on the bioactivity of BMP-2 by preparing a series of sulfated CS has been investigated by Zhou *et al.* (H. Zhou et al., 2009). The enhanced bioactivity of BMP-2 was attributed primarily to the stimulation from 6-O sulfated CS (6SCS), while 2-N sulfonated CS (2SCS) was subsidiary group with less activation, whereas low dose of 2-N, 6-O sulfated CS (26SCS) stimulated the osteoblast differentiation induced by BMP-2 *in vitro* and the ectopic bone formation *in vivo.* An increase in chain length and sulfur content resulted in a higher alkaline phosphatase (ALP) activity. Hence, sulfated CS stimulated the proliferation of both human primary osteoblasts (OB) and the OB like stromal cell component of the giant cell tumor of bone

(GCTB) at a concentration of 100 µg.mL^{-1}, while it inhibited it at higher concentration (1000 µg.mL^{-1}) (Tang et al., 2011). Thus, sulfated CS could be used as bone repair biomaterials with the dual properties of bone induction and bone tumor inhibition.

For the development of a new fibrous tissue, it is important that neovascularization occurs at the site of bone defect. It was proven by Yu *et al.* that 26SCS could promote revascularization for tissue regeneration and thus, could be used as promising angiogenic biomaterial (Y. Yu et al., 2018). A study from Cao *et al.* used sulfated CS to develop a composite photopolymerisable hydrogel incorporating rhBMP-2 loaded 26SCS based nanoparticles as a promising bone substitute (Cao et al., 2014). This composite displayed excellent cell viability, cell adhesion and cell in the growth of human mesenchymal stem cells (hMSC). *In vitro* results showed a high ALP activity and mineralization and *in vivo* results evidenced ectopic bone formation in mouse thigh defect and rabbit radius critical defect models. Likewise, nanoparticles of thiol-modified CS sulfate have been prepared and showed a potential use as novel materials for specific delivery of Basic Fibroblast Growth Factor (bFGF) with mitogenic activity (Ho et al., 2010). In addition, the attachment of 26SCS on poly (lactide-co-glycolide) (PLGA) scaffolds exhibited a better environment for cells attachment, an improved rhBMP-2 adsorption and prolonged release process *in vitro* (Kong et al., 2014). Similarly, 26SCS modified electrospun fibrous poly(caprolactone) PCL scaffold for BMP-2 delivery improved osteoinduction (Cao et al., 2017). Furthermore, novel bioactive nanocomposite scaffolds were prepared by Ghaee *et al.* from CS, sulfonated CS and PCL nanofibers (Ghaee et al., 2017). Basically, sulfonated CS was blended with CS to improve the bioactivity of CS-based scaffolds and chopped-hydrophilic PCL nanofibers were incorporated into fabricated scaffold to mimic ECM-like structure promoting cell viability and attachment. Then, calcium-phosphate (CaP) was deposited via alternate soaking in CaP rich solution. Finally, through literature, sulfated CS derivatives are involved in different shaped biomaterials for BTE applications such as coatings on polymeric scaffolds (Kong et al., 2014), electrospun fibres-based mats (Cao et al., 2017), composite scaffold incorporating PCL nanofibers (Ghaee et al., 2017) and nanoparticles (Cao et al., 2014).

3.3. Drug delivery

Numerous drug delivery systems have been studied, such as liposomes (Zununi Vahed, Salehi, Davaran, & Sharifi, 2017), nanoparticles (B. Kumar, Jalodia, Kumar, & Gautam, 2017), prodrugs (D. Huang et al., 2018), nanofibers-based membranes (Ouerghemmi et al., 2016) and polymeric micelles (Bölgen, 2018). An increasing interest has been paid for the use of polymeric micelles as novel colloidal delivery systems that can fulfill the requirements of an ideal and versatile drug carrier (Nasri & Mirshekarpour, 2015).

Due to its pharmaceutical characteristics, such as biocompatibility, hemostasis, pH sensitivity, biodegradability and its ability to be metabolized by some human enzymes, especially lysozyme, the interest in CS and its derivatives in drug delivery applications increased in recent years (G. Huang, Liu, & Chen, 2017). Raw CS is soluble in aqueous solutions of various acids, but it has no amphiphilicity and consequently cannot form micelles. Novel water-soluble CS derivatives have been synthesized in order to overcome these drawbacks. Many works have been done to modify CS molecules by attaching long chain alkyl groups as hydrophobic moieties and sulfate groups as hydrophilic moieties for the solubilization of drugs as potential brain-targeting carrier (Yao, Zhang, Ping, & Yu, 2007) or delivery system

for chemotherapy (Jin, Mo, Ding, Zheng, & Zhang, 2014; Qu et al., 2009; C. Zhang, Qu, Sun, Wu, et al., 2008). N-alkyl-O-sulfated CS showed amphiphilic character and displayed the ability to form micelles of size around 100-400 nm (C. Zhang et al., 2003a). Indeed, the *in vivo* safety and side effects of SCS compounds were poorly reported in literature. Zhang et al. reported the LD50 value of N-octyl-O-sulfate (NOSC) administrated by i.v. and i.p. to mice were 102.59 and 130.53 mg/Kg, respectively (C. Zhang, Qu, Sun, Yang, et al., 2008) . Moreover, no intravenous stimulation, injection anaphylaxis, hemolysis and cytotoxicity were observed in the safety studies. Finally, authors concluded that tissue distribution, pharmacokinetics, excretion and safety studies were persuasive for the potential application of NOSC as drug carrier. Taxol, a water insoluble anticancer drug, has been solubilized into the polymeric micelles by physical entrapment and showed a Taxol concentration of about 2.01 mg/mL that was much higher than that in pure water. Similarly, NOSC micelles were prepared to improve the oral absorption of Paclitaxel, an antineoplastic agent that has a powerful antitumor activity (Jin et al., 2014; Mo et al., 2011, Zhang, C., Qu, G., Sun, Y., Wu, X., Yao, Z., Guo, Q., … Zhou, H. (2008). Pharmacokinetics, biodistribution, efficacy and safety of N-octyl-O-sulfate chitosan micelles loaded with paclitaxel. Biomaterials, 29(9), 1233–1241). The *in vivo* assays showed that the oral bioavailability of Paclitaxel loaded in NOSC micelles was 6-fold improved in comparison with that of an orally dosed Taxol®, the commercially available product. ~~These studies suggested that N-alkyl-O-sulfate CS may be used as an efficient drug carrier.~~

Murali *et al.* have prepared sulfonated CS nanoparticles loaded with Amphotericin B, a polyene antifungal produced by *Streptomyces nodosus*, in order to treat intracellular *Candida glabrata* infections (Murali et al., 2018). The prepared particles, which the size was approximately 300 -310 nm, were hemocompatible and compatible with RAW 264.7 cells. Besides, thanks to sulfate groups, the nanoparticles exhibited the best antifungal activity. Thus, sulfated CS derivatives could be used as therapeutics for treating chronic and stubborn infections associated with intracellular pathogens (Murali et al., 2018).

Moreover, Sulfonate CS derivatives have been used as novel polymeric absorption enhancer for the oral administration of macromolecules, such as heparin. A study has been investigated on N-sulfonato-N,O-carboxymethyl CS (SNOCC) as a potential intestinal absorption enhancer of Reviparin, a low MW heparin which is an anionic polysaccharide finding clinical application as an efficient antithrombotic agent compared to unfractionated heparin (Thanou, Henderson, Kydonieus, & Elson, 2007). SNOCC was prepared at 3 different viscosity grades 20 (SNOCC-20), 40 (SNOCC-40) and 60 (SNOCC-60) cps and the results showed that SNOCC-40 and SNOCC-60 enhanced both permeation and absorption of Riveparin across intestinal epithelia.

3.4. Antiviral

Human immunodeficiency virus type-1 (HIV-1) is the causative retrovirus of acquired immune deficiency syndrome (AIDS) and is a major public health issue. It has been estimated that in 2016, there were 36.7 million HIV/ AIDS carriers globally, according to the Joined United Nations Program on HIV/ AIDS (UNAIDS) (Deeks, Overbaugh, Phillips, & Buchbinder, 2015; “UNAIDS DATA,” 2017; “World Health Organization (WHO),” 2017). Different kind

of anti-HIV-1 agents have been developed but anti-AIDS treatment is limited due to the emergence of resistant viruses, cross-resistance to drugs and cell toxicity (Hung, Lee, Chen, Chan, & Chen, 2014; S.-A. Lee et al., 2010; Marconi et al., 2008; Mulu, Liebert, & Maier, 2014; Salehi et al., 2018). Hence, the research has turned to the use of natural bioactive materials and their derivatives in order to make new anti-HIV therapeutics with higher activity and fewer side effects (Adamson & Freed, 2009; Danial & Klok, 2014; Narayan, Rai, & Tewtrakul, 2013; Schaeffer & Krylov, 2000; Vo & Kim, 2010). Interestingly, sulfated polysaccharides have been reported by several authors to be effective toward the inhibition of HIV activity (Budragchaa et al., 2015; Ngo & Kim, 2013; Wijesekara, Pangestuti, & Kim, 2011). Some works reported the use of CS derivatives for therapy purposes, based on their beneficial biodegradability and non-toxicity properties, and suggested that sulfated CS derivatives could present antiviral activity. Indeed, Sosa *et al*. prepared N-carboxymethylchitosan N,O-sulfate from N-carboxymethylchitosan via a random sulfonation reaction, that competitively inhibited the HIV-1 reverse transcriptase and binding to human CD_4^+ target cells and that of Rauscher murine leukemia virus (RLV) in murine fibroblast (Sosa, Fazely, Koch, Vercellotti, & Ruprecht, 1991). Nishimura *et al.* investigated the influence of sulfonate or sulfate groups position and suggested that the selective sulfonation at O-2 and/or O-3 afford a potent antiviral agent showing a much higher inhibitory effect on the infection of AIDS virus than that by the known 6-O-sulfated derivative (Nishimura et al., 1998). Furthermore, it has been reported by Atan *et al.* that sulfated chitooligosaccharides (SCOS) III (MW 3-5 kDa) were the most effective CS derivatives for inhibiting HIV-1 replication, by blocking viral entry and virus-cell fusion probably via disrupting the binding of HIV-1 to CD4 cell surface receptor, and that the anti-HIV-1 activity was dependent on the MW (Artan, Karadeniz, Karagozlu, Kim, & Kim, 2010).

3.5. Antimicrobial

The antimicrobial activity of CS, that was discovered by Alan (Allan & Hadwiger, 1979) and Kendra (Kendra & Hadwiger, 1984), and antifungal properties have been widely explored in biomedical, food, biotechnological, agriculture and pharmaceutical industry (Braz et al., 2018; Divya, Smitha, & Jisha, 2018; Ma, Garrido-Maestu, & Jeong, 2017; Mohandas, Deepthi, Biswas, & Jayakumar, 2018; Perinelli et al., 2018; Tachaboonyakiat, 2017; Yuan, Chen, & Li, 2016; X. Zhang et al., 2017). Furthermore, scientists revealed that the antimicrobial action was likely caused by the presence of protonated amino groups on CS skeleton since they can electrostatically interact with the phosphoryl groups of phospholipid components of cell membranes (D.-S. Lee & Je, 2013; Xie, Hu, Wang, & Zeng, 2014). Some works have proved that the antimicrobial activity is dependent on MW and concentration of CS (Amato et al., 2018; Escárcega-Galaz et al., 2018; Garcia et al., 2018; Kaya, Asan-Ozusaglam, & Erdogan, 2016; Vaz et al., 2018) and on environmental parameters such as pH, temperature, salinity, etc. (Ardila, Daigle, Heuzey, & Ajji, 2017; Chang, Lin, Wu, & Tsai, 2015; Malinowska-Pańczyk, Staroszczyk, Gottfried, Kołodziejska, & Wojtasz-Pająk, 2015). However, the poor solubility of CS in water limits its applications. Thus, it is necessary to generate novel bio-functional materials via chemical modifications of CS to improve its properties (Bakshi, Selvakumar, Kadirvelu, & Kumar, 2018). Anionic polysaccharides carrying sulfate or sulfonate functions exhibited good biological properties, including antimicrobial effects (Bueno et al., 2015; Krichen et al., 2015; Patel, 2012). Sun *et al.* prepared a water-soluble sulfonated CS, through a facile chemical procedure by using 1,3-propane sultone that exhibited high antibacterial

activities against *Escherichia coli* and *Staphylococcus aureus* with the minimum inhibitory concentration (MIC) of 0.13 mg/mL and 2.00 mg/mL, respectively, and also antifungal activities against *Arthrinium sacchari* (64 mg/mL) and *Botrytis cinerea* (0.25 mg/mL) (Sun et al., 2017). Likewise, another anionic water-soluble polymer, alkylsulfonated CS, enhanced the antimicrobial effectiveness and skin compatibility, as it possessed an outstanding microorganism inhibition against fungal reference strain of *Malassezia furfur*, *Malassezia pachydermatis*, *Trichophyton rubrum*, *Trichophyton mentagrophytes* and *Candida albicans*, together with four different bacteria species of *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus and Propionibacterium acne* (H.-F. Huang & Peng, 2015). Thus, Huang *et al.* reported alkylsulfonated CS derivatives could be used for treating acne, by contributing to the restoration of the regular pH of adult skin and by providing a non-conducive environment for the growth of microorganism strains (H.-F. Huang & Peng, 2015). Moreover, it has been proved by Zhong *et al*. that the antimicrobial activity was affected by MW of CS derivatives, since their antimicrobial activity varied reversely with MW (Zhong, Li, Xing, & Liu, 2009).

3.6. Antioxidant

Reactive oxygen species (ROS) are generated by living organisms as a result of normal cellular metabolism. They include free radicals in the forms of superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), and non-radical molecules like hydrogen peroxide (H_2O_2). During environmental stresses, they can cause damage to a wide range of essential biomolecules, such as peroxidation of lipids leading to the accumulation of lipid peroxides, oxidation of proteins, enzyme inhibition, damage to nucleic acids, activation of programmed cell death pathway, etc. (Ghosh, Sarkar, & Paul, 2016; Kardeh, Ashkani-Esfahani, & Alizadeh, 2014; Tabima, Frizzell, & Gladwin, 2012). ~~Thus,~~ Oxidative stress is believed to be a primary factor in various degenerative diseases, as cancer, rheumatoid arthritis, atherosclerosis and Alzheimer's disease, as well as in the normal process of ageing (Cheignon et al., 2018; Ghosh et al., 2016; Kovacic & Somanathan, 2012; Srivastava & Dubey, 2011). Thus, researchers have turned to antioxidant agents, especially natural ones, which can scavenge or prevent the production of ROS and activate a battery of detoxifying proteins. Polysaccharides, such as CS, have attracted a big interest because of their chemical structure that contains a high content of amino groups (Ai, Wang, Xia, Chen, & Lei, 2012; Aytekin, Morimura, & Kida, 2011; Ying, Xiong, Wang, Sun, & Liu, 2011). It has been demonstrated that the antioxidant activity is related to the presence of free amino groups and hydroxyl groups in the CS backbone (Je, Park, & Kim, 2004). Besides, many works have dealt with sulfated or sulfonated polysaccharides preparation for antioxidant activity purposes, especially those based on CS, as they could scavenge superoxide anion and hydroxyl radicals, and reduce power (Seedevi et al., 2017; Xing et al., 2004; J. Yang et al., 2015). Several studies attempted to investigate the relationship between physicochemical characteristics of SCS and antioxidant property. As a matter of fact, high MW and high sulfate content in CS derivatives exhibited an antioxidant activity (Xing, Liu, et al., 2005). The scavenging activities of sulfated CS on superoxide and hydroxyl radicals were more pronounced than that of CS. Besides, low MW CS sulfate had more effective scavenging activity than that of high MW CS sulfate (Xing, Liu, et al., 2005). The antioxidant activity of different regioselective sulfated CS has been investigated by Xing *et al.* (Xing, Yu, et al., 2005). A comparison has been made between a sulfated CS on positions 2, 3 and 6 (HCTS); a sulfated CS on position 3 and 6 (TSCTS); a sulfated CS on position 6 (SCTS) and a sulfonation on position 3 (TCTS). The scavenging activity of superoxide radical was found to be in the order of HCTS

> SCTS > TCTS > TSCTS, all kind a sulfated CS were efficient in the reducing power, especially TSCTS, and a considerable ferrous ions chelating potency has been observed for TSCTS and TCTS, making them good candidates for pharmaceutical and food industries (Xing, Yu, et al., 2005). These results allow concluding that the sulfonation on position 6 is quite important for antioxidant activity and that the sulfonation on amino groups could help to enhance this property. These results were consistent with those of recent study, that highlighted the slight increase of scavenging capacity when DS significantly decreased (J. Yang et al., 2015). When DS gradually decreased and unsubstituted amino groups rose, the scavenging activity of sulfonated derivatives did not show a significant increase, which could be related to the electrostatic repulsion occurring between negative charges (J. Yang et al., 2015). In another study, Huang *et al.* reported that hydroxyethyl CS sulfate (HCS) could scavenge 2, 2-diphenyl-1-picrylhydrazyl (DDPH) (33.78% at 2.5 mg/mL) and carbon-centered radicals (67.74% at 0.25 mg/mL) confirming that HCS can be considered as effective antioxidant compound to delay lipid peroxidation. On contrary, CS sulfate did not exhibit any scavenging activity against hydroxyl radicals but rather increased its generation, which is controversial to the other works (R. Huang, Mendis, & Kim, 2005). Thus, the antioxidant property of sulfated CS in relation to sulfated and amino groups are still ambiguous.

3.7. New trends

Additionally to the biomedical applications discussed in the previous sections, several recent studies reported the use of SCS derivatives in innovative applications of cellular biology applied to medicine, such as neural differentiation (Doncel-Pérez et al., 2018). Indeed, sulfated CS has been used by Ding *et al.* as candidate for inducing and differentiating Embryonic Stem Cells (ESCs) into nerve cells, which is the key for the development of neural drugs for nerve repair and regeneration (Ding et al., 2014). This study showed that the induced neural differentiation was dependent on both sulfation site and DS, as compared with 2-N, 6-O sulfated CS and with 3, 6-O sulfated CS, selectively modified 6-O sulfated CS more efficiently promoted the neural differentiation of ESCs and the activity rose with increasing DS. However, sulfation site seemed to have more influence compared to DS (Ding et al., 2014). Furthermore, very recently, sulfated CS derivatives were reported to exhibit immunostimulatory effects on RAW 264.7 mouse macrophages (Y. Yang et al., 2018).

Workers have compared the immunostimulatory activity between α- and β-sulfated CS with different molecular weights. α- CS, the most extensively studied CS, is found in crab and shrimp while β-CS is primarily obtained from squid pens. On contrary of α-CS, β-CS displays parallel orientation of its polysaccharidic backbone and consequently a less dense intermolecular hydrogen bonds network, making it more soluble and more reactive than α-CS with the same molecular weight. Therefore, sulfated β-CS significantly enhanced the production of nitric oxide (NO) in a dose-dependent manner, prostaglandin E_2, tumor necrosis factor (TNF)-α, interleukin-6 and interleukin-1β at the levels of transcription and translation, compared to sulfated α-CS (Y. Yang et al., 2018). Consequently, sulfated CS could be a potential candidate as immunostimulator especially as a vaccine adjuvant. Finally, another very recent study from Luo *et al.* reported the preparation of a composite hydrogel formed through the Schiff-base reaction between the aldehyde of oxidized konjac glucomannan (OKGM) and the amine of carboxy CS sulfate (CMSS) (Luo et al., 2018). The obtained hydrogel benefited from excellent physicochemical and biological properties, such as short gelation time,

good swelling ability, appropriate water evaporation rate, excellent hemocompatibility and cytocompatibility on NIH-3T3 cells. These characteristics make CS derivative an appropriate candidate as a wound dressing for injured skin treatment.

In conclusion, as mentioned above, SCS present a wide range of biological activities and present high potential for many therapies. Therefore, depending on its applications, SCS can be used in different shapes, such as in solution, as micelles, as devices coatings, electrospun mats, in composite materials, hydrogels as summarized in figure 6.

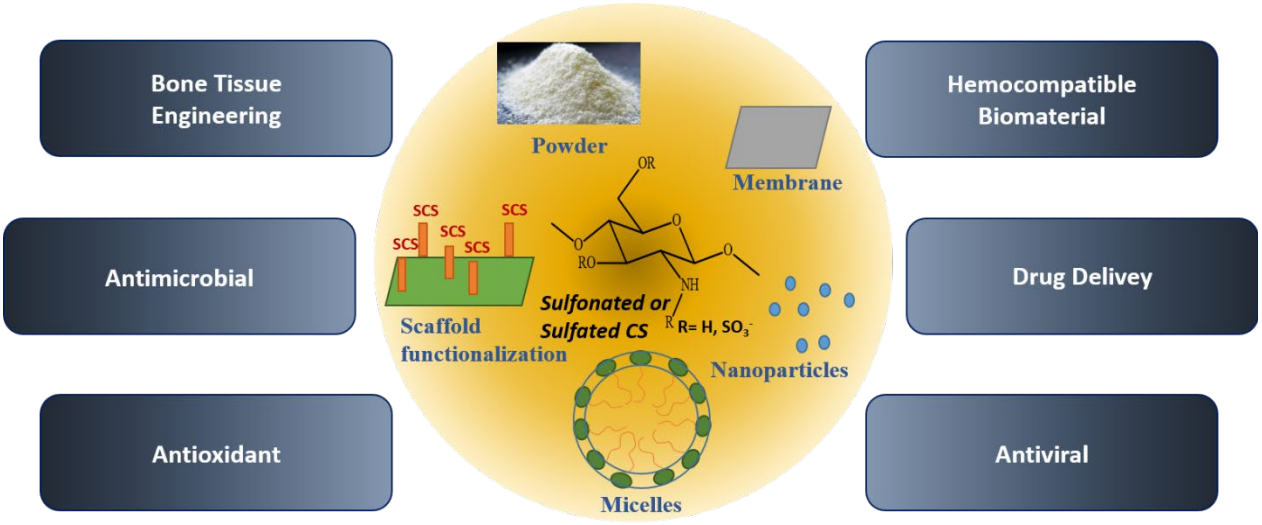

Figure 6. Several examples of SCS shapes and their biomedical applications

Table 2 summarizes the effect of different sulfonating reagents on regioselectivity, the physicochemical and biological properties and highlights potential biomedical applications of CS derivatives.

622 **Table 2.** A summary of sulfonating reagents' effects on regioselectivity, the physicochemical and biological properties and their biomedical applications

Reagent	Position	Physicochemical property	Biological property	Application
SO_3.DMF	N,O-	MW = 22 000 g.mol^{-1} ; DS = 1.7 (Shigehiro Hirano et al., 1985)	Anticoagulant and lipoprotein lipase activity	Blood contact application (S. Hirano et al., 1991; Shigehiro Hirano et al., 1985; Ramasamy et al., 2017)
		MW = 56 200 – 139 000 g.mol^{-1} ; %S = 13.19 – 14.57 in DCAA Or MW =71 200 – 13 400 g.mol^{-1} ; %S = 13.45 – 14.52 in FA	Scavenge of superoxide and hydroxyl radicals, reducing power and slight chelating activity	Antioxidant (Xing, Liu, et al., 2005)
SO_3.pyridine	N,O-	MW = 18 100 – 54 400 g.mol^{-1} ; DS = 1.65 – 2.46 (J. Yang et al., 2013)	Anticoagulant	Blood contact application (Whistler & Kosik, 1971; J. Yang et al., 2013)
		MW = 16 000 g.mol^{-1} ; DS = 1.69	Antiretroviral	Anti-HIV-1 agent (Nishimura et al., 1998)
		MW = 124 000 g.mol^{-1}; %S = 14.7%; easy soluble	Free radical scavenging, chelation and reduction of Fe^{3+}	Antioxidant (Xing et al., 2004; Xing, Yu, et al., 2005)
	N-	DS = 0.25 – 0.32	Absorption and permeation enhancer	Oral delivery of LMWH reviparin (Thanou et al., 2007)
$HClSO_3$	N,O-	MW = 5 120 – 26 200 g.mol^{-1} (Suwan et al., 2009); Mv = 5 210 – 7 900 g.mol^{-1} DS = 0.18 – 0.81 (R. Huang, Du, & Yang, 2003)	Anticoagulant	Blood contact applications (R. Huang, Du, & Yang, 2003; R. Huang, Du, Yang, et al., 2003; Suwan et al., 2009; Vongchan et al., 2002)
		DS= 0.76 (Ghaee et al., 2017)	Increase apatite mineralization, high ALP activity, mineralization and *in vivo* ectopic bone formation ability Bone induction and bone tumor inhibition	Bone tissue engineering (Cao et al., 2017; Ghaee et al., 2017; Kong et al., 2014; Tang et al., 2011)
	O-	DS = 0.66 in DMF DS = 0.96 in DMF/ water (97/3 V/V)	Anticoagulant, low protein adsorption and platelet adhesion inhibition	Blood purification (Xue et al., 2013)
		DS = 1.13 – 1.67 under homogeneous conditions or DS = 0.86 – 1.67 under	Oral absorption of paclitaxel	Drug delivery systems (Mo et al., 2011; Qu et al., 2009; Yao et al., 2007; C. Zhang et al., 2003a, 2004; K. Zhang et al., 2010)

		non-homogeneous conditions (K. Zhang et al., 2010)		
$HClSO_3$	O-	DS = 48.44 %	Ability of copper ions chelation	Artificial multinuclear phosphodiesterase (Xiang et al., 2010)
		MW = 32 800 $g.mol^{-1}$; %S = 9.58% Water soluble	Inhibition of both, viral adsorption and reverse transcription	Anti-HIV-1 agent (Sosa et al., 1991)
FFSA	N-	%S = 3.5% (Lima et al., 2013)	Platelet adhesion and activation Anticalcification and antithrombogenic Low protein adsorption Anticoagulant	Blood contact applications (Amiji, 1998; Campelo et al., 2017, 2016; Lima et al., 2013; Liu et al., 2004)
Sulfobenzoic acid cyclic anhydride	N-	Zeta potential: -6.06 ± 1.22 mV; DS = 1.12%; swelling behavior	High hemocompatibility	Blood contact applications (Shelma & Sharma, 2011)
Me_3N-SO_3	N,O-	MW = <1 000 - 10 000 $g.mol^{-1}$	Inhibition of HIV-1 replication	Anti-HIV-1 agent (Artan et al., 2010)
		DS = 0.76	Prolyl endopeptidase inhibitory activity	Blood contact applications (Je et al., 2005)
	N-	MW = 20 300 – 47 600 $g.mol^{-1}$; DS = 0.83 - 1.52; crystallinity reduced (J. Yang et al., 2015)	Scavenge of superoxide and hydroxyl radicals and reducing power	Antioxidant (Xing, Yu, et al., 2005; J. Yang et al., 2015)
1,3-propane sultone	N,O-	MW = 31 474 $g.mol^{-1}$; %S = 8.07% (Sun et al., 2017)	Antimicrobial and skin tissue compatible	Bactericide and fungicide substitute (H.-F. Huang & Peng, 2015; Sun et al., 2017)
	N-	%S = 0.82 – 3.18%; crystallinity reduced; amphoteric character	Proliferation of HDF	Tissue engineering (Jung et al., 2007)
BZ1S/ BZ2S	N-	DS = 0.11 – 0.69 (BZ1S) and 0.26 – 0.41 (BZ2S); Zeta potential = -42 to -19 (BZ1S) and -31 to -36 (BZ2S); polyampholytic derivative	Anticoagulant	Blood contact applications (Ouerghemmi et al., 2018)

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4. Conclusion and Future Perspectives

Literature reports that chemical modification of CS by sulfate or sulfonate groups may potentially lead to a wide range of reaction compounds due to the presence of three possible reacting sites on the polymer repeat units. However, chemists have defined the experimental conditions for controlling more or less accurately the regioselectivity of the reaction and the degree of substitution of the CS derivatives. SCS presents different physicochemical properties compared to parent CS, mainly due to the resulting polyampholytic character. This review displayed that such features widely expanded the biological properties of CS, and enlarged its domain of applications. Indeed, through the literature published since the early 1990's, hemocompatible, anticoagulant, anticalcifying, angiogenic, antiviral, antimicrobial, antioxidant and drug carrier properties were attributed to SCS. Depending on the targeted applications, these compounds can be used in solubilized form, as micelles, films, coatings, nanowebs, hydrogels and present high potential for cardiovascular, drug delivery, bone reconstruction, blood purification, cancer treatment and neural differentiation applications. This review article has also highlighted that medical applications did not depend mainly on substitution position, as different biological properties have been noticed for the same chemical modification position. Moreover, one specific biological property could be reached from different SCS derivatives.

In the last decade, tremendous progress has been achieved in the field of tissue engineering where innovative and efficient solutions for tissue regeneration have been investigated. One of the key challenges is to develop the optimal scaffold, which mimic the mechanical and functional properties of the extracellular matrix (ECM) of those tissues to be regenerated. Electrospinning, as a simple, inexpensive, versatile process, has been demonstrated to be a powerful tool for fabricating tissue-engineering scaffolds with a high surface-to-volume ratio and ECM-mimicking structures. Sulfonated CS, as a water-soluble anionic chitosan derivative, exhibited diverse biological properties (antiviral, anticoagulant, antimicrobial and osteogenic activity) besides their better processability in scaffolds fabrication. These would make them good candidates for the tissue engineering application, including vascular prosthesis, wound dressing and bone/cartilage tissue engineering.

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