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Thiolated Chitosan: A Promising Strategy for Improving the Effectiveness of Anticancer Drugs

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ABSTRACT

Anticancer drugs have intrinsic physicochemical properties which have to battle the resistance mechanisms of cancer cells countering their effectiveness. These drawbacks are solved by employing thiolated chitosan which provides an excellent mucoadhesivity. Thus, thiolated chitosan has a membrane permeation enhancing ability and better inhibition of P-glycoprotein. As a result, anticancer drugs show an improved bioavailability and residence time. In this chapter, the synthesis and characterization of thiolated chitosan structured in delivery systems such as hydrogels, films and micro and nanoparticles is discussed as novel delivery systems for anticancer drugs.

Key words: Smart Polymers; Thiolated Chitosan; Drug Delivery Systems; Anticancer Drugs; Cancer Treatment

Abbreviations: Anticancer Drugs **(ACDs)**; Active Pharmaceutical Ingredient **(API)**; Antisense Oligonucleotide **(ASOND)**; Biopharmaceutical Classification System **(BCS);** Chitosan **(CH);** Cyclic Voltammetry **(CV);** Cysteine **(CYS); D**oxorubicin **(DOX);** Docetaxel **(DTX);** Entrapment Efficiency **(EE)**; Glutathione on the Reduced Form **(GSH)**; Glutathione on the Oxidized Form **(GSSG)**;

Methotrexate **(MTX);** Molecular Weight **(MW);** N-Acetylcysteine **(NAC);** N-Acetyl Penicilamine **(NAP);** Nanoparticles **(NPs);** P-Glycoprotein **(P-gp)**; Commercial Pcl and Self-Synthesized D-A-Tocopheryl Polyethylene Glycol 1000 Succinate Random Copolymer **(PLA-PCL-TPGS);** Polymethyl Methacrylate **(pMMA)**; Paclitaxel **(PTX)**; Thiolated Chitosan **(TCH);** Thioglycolic Acid **(TGA)**; Homocysteine Thiolactone **(HT);** N,N,N-Trimethyl-Chitosan **(TM-CH)**; Sodium Tripolyphosphate **(TPP)**; Transfection Rate **(TR)**; 2-Imiothiolane **(2-IMT)**; 4-Mercaptobenzoic Acid **(4-MNA)**; 4-Thio-Butylamidine **(4-TBA)**; 6-Mercaptonicotinic Acid **(6-MNA)**

INTRODUCTION

Chemotherapy is used for the treatment of intermediate and late stage of cancer disease and can be combined with radiotherapy when tumors are unrespectable with surgery [1]. However, many chemotherapeutic agents have a low aqueous solubility, especially those derived from proteins and DNA [2]. As a result, their intravenous administration must be limited [3]. On the other hand, the oral administration of these drugs is preferred since this way guarantees a better patient compliance and renders few side effects. However, the oral pathway presents some shortcomings such as the passage of the drug through the acidic stomach ambient, presence of proteolytic enzymes in the gastrointestinal tract, and a first-pass metabolism. Further, in some cases, a non-homogeneous biodistribution and a short half-life of drugs having a low bioavailability such paclitaxel, doxorubicin, docetaxel, and tamoxifen is obtained (bioavailability of 5-20%). Therefore, for those anticancer drugs **(ACD)**, the oral administration might not be the first option [2].

On the other hand, cancer cells have also developed resistance to ACD. For instance, the efflux pumps (i.e., P-gp) avert the therapeutical success because they induce a high serum concentration of ACD [4]. These P-pg play an essential role for the transportation of vincristine, vinblastine, daunorobucin, epirubicin, etoposide, teniposide, methotrexate, 6-mercaptopurine, gemcitabine, and mitomycin C [5].

Several drug delivery systems **(DDS)** have been created to ameliorate the previously mentioned issues. These DDS are based on natural, or synthetic polymers, which in turn, are capable to accumulate on tumor cells due the enhanced permeability, retention effect, and mucoadhesiveness, resulting in the net improvement of drug permeability [6]. Chitosan appear to be the ideal polymer for DDS due its distinctive properties such as great biocompatibility, biodegradability, and mucoadhesivity. Further, it is relatively easy to incorporate the desired functional groups in the polymer backbone.

Chitosan derivatives having thiols groups **(-SH)** on their surface are also known as thiomers [7] and have powerful features such as *in-situ* gelling, excellent mucoadhesivity, efflux pump-inhibiting capability, cell permeation, and pDNA protection against degradation [8]. Further, they form more stable complexes by formation of disulfide bonds between chains [9]. Likewise, these mucoadhesive drug delivery carriers provide advantages such as: (i) An improved bioavailability

of drugs by increasing the residence time in the mucosa due to the lack of absorption of the polymer; (ii) a lower frequency of administration by controlling drug release in the gastrointestinal tract; (iii) protection from the acidic environment of the stomach and evasion of enzymatic degradation; (iv) cost effectiveness, and reduction of dose-related adverse effects by improving the therapeutic performance of drugs; (v) target specificity to particular tissue sites by placing drugs directly into the mucosal tract; and (vi) they exhibit a large patient compliance as compared to parenteral injections [10]. Consequently, the purpose of this chapter is to discuss the synthesis, characterization, and gelling properties of DDS composed of thiolated chitosan.

SYNTHESIS OF THIOLATED CHITOSAN

The addition of thiol groups to CH extends the stability and adhesion capability of various dosage forms as compared to unmodified CH on different mucosal tissues [11]. The synthesis of TCH implies the use of several compounds such as 6-MNA, CYS, 2-IMT, NAP, 4-MNA, NAC, GSH and TGA. All these compounds lead to the formation of amide or amidine bonds depending on the selected ligand (Figure 1) [12]. These ligands are attached to CH forming covalent bonds with the amine groups located on the C-2 of the glucosamine subunit [12].

The amide bonds are originated by coupling an activated o-acylurea formed between the carboxylic acid group of the ligand (TGA, NAC) and the carbodiimide [13]. In this process, cysteine should be avoided since it causes unintended side reactions. Particularly, the N- or C- protected cysteine is used instead [12]. Since in this procedure thiol groups can be oxidized, the reaction must be carried out under inert conditions, or pH values lower than 5 [14]. Under these conditions the reactivity of the anion form of thiol groups is low, and disulfide bonds can be excluded. If the reaction takes place at a pH >5, agents such as dithiotreithol or borohydride should be added at the end of the reaction in order to reduce the formed disulfide bonds (a two stage-reaction) [15]. On the other hand, the amidine bonds can be formed by nucleophilic reaction of the amino groups and imidates such as the 2-iminothiolane (Traut's reagent) [16]. Consequently, this process is preferred due to the chemical structure of the ligand which protects the thiol group against oxidation [17]. Moreover, these chitosan derivatives have a more cationic character than CH alone, whereby they precipitate at a pH between 6.5 and 7.5 depending on the resulting degree of modification in comparison to CH alone which precipitates at pH values > 6 [18].

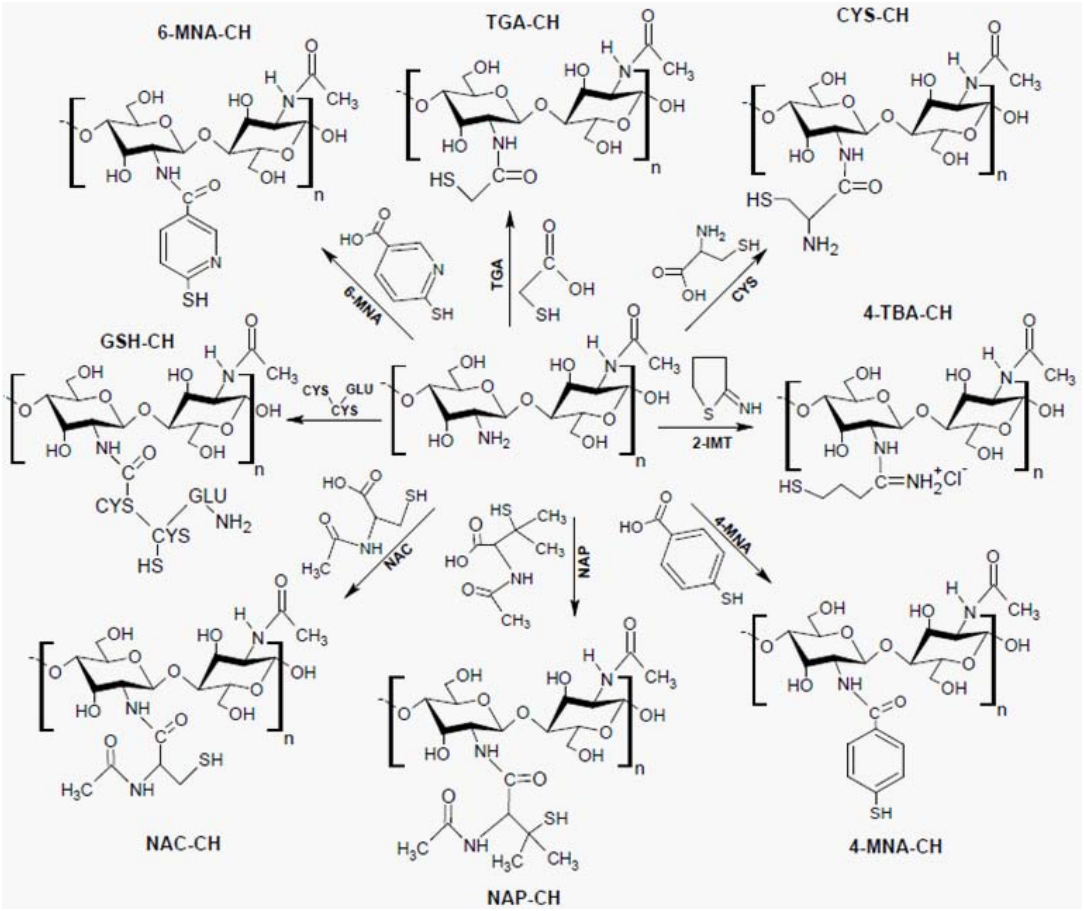

Figure 1: Schematics of the synthesis of different TCHs from CH. A: 6-MNA-CH, B: TGA-CH, C:CYS-CH, D: GSH-CH, E: 4-TBA-CH, F: NAC-CH, G:NAP-CH, H: 4-MNA-CH.

The reaction conditions of representative TCH is described as follows:

• **6-MNA-CH:** In this case, the reaction is carried out at a pH of 5.0 for 7h using carbodiimide as mediator and a CH:6-MNA ratio of 1:2.5. After 7h of reaction, the tris (2-carboxyethyl) phospine hydrochloride is added and incubated for 30 minutes. The new TCH is considered non-toxic and showed a 30-fold increase in viscosity as compared to CH at a pH of 5.5 and 37˚C. Additionally, this TCH can react in a non-pH-dependent way [19].

• **TGA-CH:** In this reaction, the attachment of TGA on CH is conducted at a pH of 5.0 and at a CH:TGA 1:1 ratio, using cabodiimide at a 50 mM concentration. The mucoadhesivity and swelling of this conjugated at a pH of 6.0 exhibited a 3.1-fold and 2.3-fold increase as compared to the parent CH [13].

• **CYS-CH:** The attachment of CYS on the backbone of CH is mediated by carbodiimide at different concentrations (30, 40 and 50 mM). The immobilization of thiol groups was achieved with

the increase of cabodiimide concentration. As a result, the mucoadhesivity showed a major enhancement, but the swelling behavior of these derivates was independent of degree of thiolation at a pH of 6.8. Further, the derivative having the most substituted thiol groups showed the slowest release of the highly soluble metformin.HCl [20].

• **GSH-CH:** This derivative is produced when GSH moieties are coupled on CHI using cabodiimide and N-hydroxysuccinimide at a concentration of 200 mM. The reaction was carried out for 7h atambient temperature and pH of 6.0. This derivate showed a 9.9-fold, 5.5-fold and 4.9-fold increase on total work of adhesion, adhesion time and permeation-enhancing effect, respectively as compared to CH [21].

• **4-TBA-CH:** The production of this TCH involves the use of 2-iminothiolane.HCl and CH at a 2.5:1 ratio. The pH of this reaction is adjusted to 6.0 and the reaction is continued for 24h under continuous stirring. Films of this conjugate in combination with GSH had a 3-fold increase in permeation as compared to CH alone. Further, this derivative had a sustained release of cefadroxil. Furthermore, mucoadhesivity of this TCH showed a pH-dependent profile. Thus, mucoadhesivity is larger at a pH between 3.0 and 5.0 [22].

• **NAC-CH:** In this conjugation, the carboxilyc acid groups of NAC are activated with carbodiimide for 20 minutes. Subsequently, this activated NAC is added to the CH solution and the coupling reaction is performed for 6h under constant stirring. This TCH shows a 50-fold increase in the residence time on mucosa as compared to CH. Additionally, tablets made of TCH exhibit a total work of adhesion 8.3-fold higher than CH on mucosa and a limited swelling at a pH of 6.8 (only 5%, after 2 h) [23].

• **NAP-CH:** In this reaction, CH and NAP reacts at a1:1 ratio and pH of 5.0 for 3h at room temperature. Coupling of NAP was assisted with cabodiimide (200mg) and new TCH has a slightly mucoadhesion compared to the CYS-CH conjugate due to strong hydrophobic interactions of the acetyl and methyl groups with mucin. Moreover, tensile studies revealed a 7-fold increase in mucoadhesion of tablets made of this thiomer as compared to CH tablets. Further, this thiomer tablets are more soluble in 100 mM acetate buffer as compared to those of CH [24,25].

• **4-MNA-CH:** This derivative is synthesized using carbodiimide as a coupling agent. This thiomer exhibits a 60-fold higher mucoadhesivity as compared to CH. The solution viscosity showed a 2974-fold and 4487-fold increase as compared to CH after 24 and 48h, respectively. Furthermore, 4-MNA has a pKa value of 6.8, showing a full reactivity at intestinal pH [26].

All the previously mentioned reaction conditions and ligand type showed that the reaction conditions have a major effect on the storage, stability, *in-vivo* performance and reactivity of TCH [12]. These compounds have a pKa between 8 and 10, indicating that at physiological conditions the anionic form of thiol groups is less prevalent and hence less reactive. As a result, a much lower quantity of disulfide bonds within the thiomers and a lower amount of cysteine-substructures in the biological materials is produced [14].

Further, it is also possible to attach thiol moieties in a non-ionic polymer in order to replace its hydroxyl groups. For instance, thiolation has been conducted on hydroxyethylcellulose in a reaction mediated by N-bromosuccinimide-triphenylphosphine in lithium bromide N,N-dimethylacetamide. As a result, an intermediate compound named as bromo-hydroxyethylcellulose is formed, which is later attacked by thiourea leading to the incorporation of thiol groups on the polymer backbone. The above mentioned procedure can also be employed on CH when the amino groups are required for further modifications [27].

DETERMINATION OF THE DEGREE OF THIOLATION

The quantification of substituents (i.e., thiol groups) can be carried out by several methods. The most wide spread technique involves UV/VIS spectrophotometry and is based on the specificity of the Ellman's reagent [5, 5′-dithio-bis (2-nitrobenzoic acid)] for the thiol groups (reaction time of 2h). This reaction takes place in solution to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid product **(TNB^{2+})** (Figure 2a). The latter renders a bright yellow solution having a high molar extinction coefficient in the visible spectroscopic range, reaching its maximum absorption peak at a wavelength between 410 and 420 nm. The recorded absorbance is then interpolated on a calibration curve previously built using cysteine-HCl as the primary standard. Nevertheless, this reaction is unstable and degrades over time, and must be carried out at pH values > 7.3, preferably from 8.0 to 8.5.Otherwise, the accessibility of the Ellman´s reagent towards the thiol groups in the cross-linked polymer is limited due the poor solubility of TCH [28,29]. Moreover, this method enables the quantification of thiol groups with or without a previous reduction of disulfide bonds with borohydride [30].

On the other hand, Bravo-Osuna and collaborators developed an alternative method based on the classical modified iodine titration. In this case, TCH is solubilized in a buffer solution (pH = 2.7) followed by the addition of the iodine and starch solutions. This mixture is then allowed to stand in the darkness for 24h at room temperature. This process implies the reaction of iodine with thiol groups causing their oxidation, and the excess of iodine interacts with the amylase chain of starch forming a blue complex having a maximum absorbance at the wavelength of 560 nm (Figure 2b). The quantification of the substituted thiol groups is then obtained by interpolation from a calibration curve previously built using cysteine-HCl as the primary standard. Modifications of this method allow for the quantification of thiol groups located on the nanoparticle surface [31].

On the other hand, the Nuclear magnetic resonance of protons **(^{1}H-NMR)** technique can be employed to determine the amount of free thiol groups. Thus, it is necessary to integrate the signals of protons 2, 3, 4 and 5 and the proton signal attributed to the thiol group. Subsequently, the area of the -SH proton signal is divided by the sum of all protons areas [32,33].

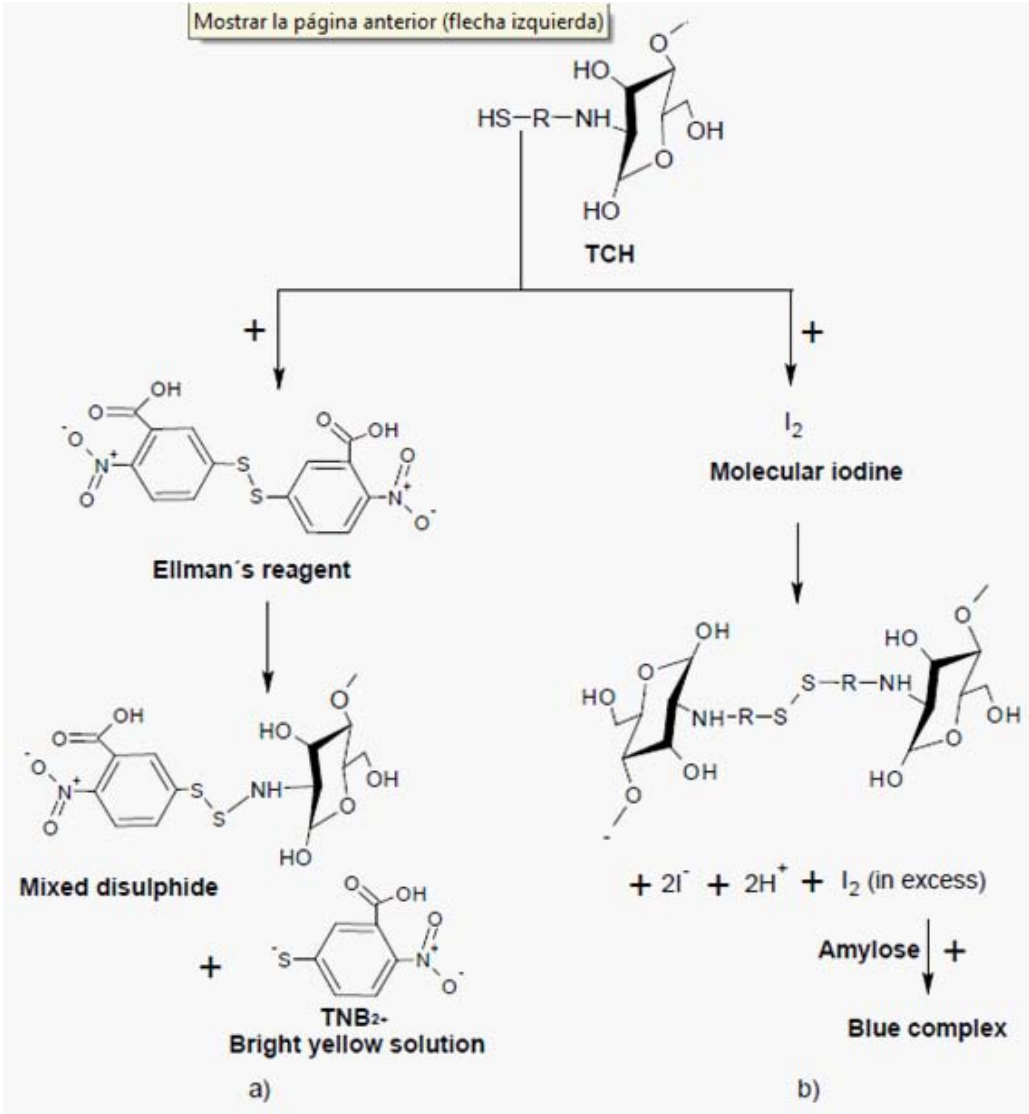

Figure 2: Reactions involved in the quantification of thiol groups. a) Ellman's reaction, b) Iodine reaction. R = thiolated ligand.

S-PROTECTION OF THIOLATED CHITOSAN

TCH has a low reactivity and is unstable in solution since thiol groups could be oxidized (i.e., at pH values >5) [34]. This issue can be overcome by using protective substructures such as mercaptopyridines producing a "second generation of thiomers" or "preactive thiomers" [35]. In this scenario, the formation of these mucoadhesive polymers is achieved in a two-stage process. First, TCH is generated as previously mentioned followed by the attachment of the mercaptopyridine moiety through disulfide linkages [36]. These new compounds can be

obtained in a broad pH range, and the aromatic moiety is easily released leaving the thiol groups unbounded [37]. The former technique was derived from covalent chromatography, where proteins, or peptides are covalently linked to thiol bearing resins (thiopropylsepharose) [36,37]. Furthermore, ligands such as mercaptonicotinamide and mercaptonicotinic acid must be used instead of mercaptopyridine due to safety reasons [34,38].

THE KEY IMPROVED PROPERTIES OF TCH

Mucoadhesiveness

Several phenomena such as wetting, diffusion, adsorption, generation of electrostatic charges, fracture tendency, and mechanical-interlocking are recalled to explain how mucoadhesion occurs [10]. Likewise, the formation of non-covalent bonds, such as hydrogen bonds, Van der Waals' forces, ionic interactions, and the physical interpenetration effects between the polymer chains and mucus play an important role in mucoadhesion [39].

Mucoadhesion is the main enhanced property of TCH as compared to unmodified CH. This is explained by the formation of covalent and disulfide bonds between the polymer and the cysteine rich regions of mucus proteins (Figure 3) [40]. These bonds are stronger than the electrostatic interactions generated with CH alone [41]. Further, these bonds are available for the thiol/disulfide exchange reactions, or simple oxidation processes of the free thiol groups [42]. For instance, TH-CH and TM-TH-CH display a 3.67and 6.33-fold stronger mucoadhesion as compared to the unmodified CH at a pH of 1.2 [43].

Furthermore, the preactivated thiomers can form stable ester bonds with the residual aspartic and glutamic acids of mucus. Moreover, thiomers can interact with the proline substructures due to the presence of electronegative nitrogen atoms. Likewise, these thiomers have a nitrogen atom capable to accept electrons that interact with the donor electrons from the oxygen atom linked to the carbonyl group of asparagine and glutamine present in mucus. Since thiomers have free thiol groups besides disulfide bonds, they could link to the cysteine rich regions of mucus leading to the formation of new disulfide bonds [44]. As a result, TCH could increase the residence time of ACDs reducing dose frequency and boosting patient-compliance [36,39].

Permeation Enhancement Capability

This phenomenon occurs due to the inhibition of the enzyme named as tyrosine phosphatase responsible for the dephosphorylation of tyrosine subunits of occludin, leading to tight junctions opening (Figure 3) [45,46]. Thus, GSH is able to phosphorylate and cause a direct occlusion, but GSH is rapidly oxidized to GSSG on the mucus surface limiting the duration of the permeation effect [8,47]. TCHs cause a shift in the GSH/GSSG balance favoring the GSH production, resulting in a reversible tight junction opening. Moreover, the addition of GSH to the thiolated polymer improves their permeation ability [48]. Further, the degree of thiolation plays a fundamental role since a higher thiolation degree implies a higher permeation capability [47].

The permeation phenomenon was studied by Dünnhaupt and collaborators who evaluated the effect of S-protected TCH and TCH on permeation of the FD4 hydrophilic macro molecule. They found that the S-protected derivatives showed a1.3-fold enhancement in permeation as compared to plane thiomers on CaCo-2 cells, and a 2-fold permeation enhancement as compared to unmodified CH, due to the presence of a higher amount of reactive thiol groups [38]. Further, Sakloetsakun and collaborators established a ranking in permeation capability of different TCHs as: 6MNA-CH>CYS-CH>GSH-CH>TBA-CH>TGA-CH> NAC-CH [49].

Inhibition of Pg-p

Thiomers possess the ability to inhibit P-gp by changing the fluidity of the cellular membrane, decreasing the amount of P-gp ATPase, and by down regulation of its expression [50]. This is due to the formation of disulfide bonds between thiomers and cysteine residues present in the two domains of P-gp [42] (Figure 3), by blocking the allosteric site of this transporter, which is crucial for moving drugs outside of cell [51].

For instance, Trapani and collaborators evaluated the P-gp inhibition capacity of CH, GCH and their thiolated derivates (conjugated with NAC or GSH) using rhodamine-123 on CaCo-2 cell mono layers. Results showed a major inhibitory effect for thiolated derivatives, especially for GCS-NAC, and GCS indicating a potential use as carriers for oral delivery of class III and IV drugs according to the BCS [52]. Further, Chen and collaborators found that TGA-CH and the S-protected derivate have an inhibitory effect on P-gp due to a decrease in plasma membrane fluidization and reduction of the P-gp ATPase activity [53].

In another study, Schmitz and collaborators evaluated the effect of P-gp rhodamine 123 substrate on the absorption of drugs by assessing the permeation effect as compared to FD4 in rat intestine and CaCo-2 monolayers of conjugated N-acetyl-chitosan having a different molar mass (i.e., 150, 400 and 600 kDa). Three conjugates showed a low cytotoxicity and the TCH of 400 kDa showed a 1.5-fold enhancement of the Rho-123 absorption, representing an easily accessible tool for the oral administration of this P-gp substrate [23].

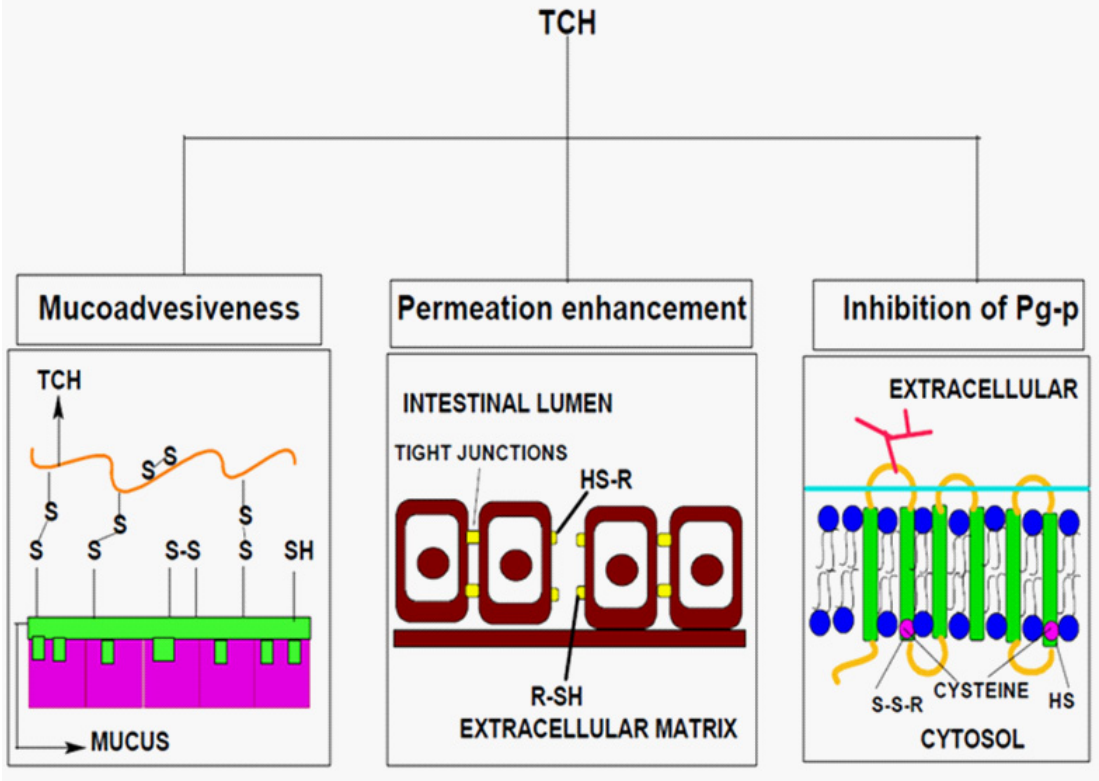

Figure 3: The key improved properties of TCH.

THIOLATED CHITOSAN-BASED DRUG DELIVERY SYSTEMS

Hydrogels

Even though the rapid clearance of drugs is the limiting factor for the efficacy of cancer treatment, it is accepted that the increase in the viscosity of the carrier can prolong the residence time of the drug on the application site [46]. For instance, TCH could form rigid hydrogels due to the formation of inter and/or intra-molecular disulfide bonds [12]. Particularly, Saboktakin and collaborators produced hydrogel films of acetyl-polyamidoamine-TCH having a large load of letrozole (i.e., 31% w/w in films) using a casting method. Thus, the LTZ release reached 19% after 15h at different pH values and a complete release in an appropriate *in-vivo* environment occurred by erosion of the biodegradable film. Conversely, the synthetic films were not able to produce local inflammatory responses, indicating a safe system for a localized chemotherapy on breast cancer cells [54].

In another study, hydrogel oral formulations composed of TGA-CH and GSH showed a 3.72-fold enhancement of the AUC of leuprolide in male Sprague Dawley rats and ~4-fold bioavailability increase as compared to the subcutaneous injection [55]. Further, TCH NPs showed a 5.2-fold, 6.9-fold, 4-fold and ~3.8-fold transport increase through the porcine nasal

mucosa, AUC, elimination half-time and maximum plasma concentration as compared to the nasal solution of leuprolide [56].

Films

Ko et al., employed CV to develop a cell chip composed of a gold electrode coated with different materials such as chitosan-6-MPA, poly-L-lysine and collagen, in order to evaluate the effect of two ACDs named as doxorubicin and cyclophosphamide. They compared their system using the MTT assay on breast cancer cells and normal cells. The cell chip coated with TCH showed superior electro-chemical performance, whereas the CV technique was more sensitive than the MTT assay on doxorubicin. Moreover, this new cell-chip was useful to assess the effectiveness of ACDs and analyze their cytotoxicity in normal cells at the same time. Consequently, this technique was important for the cell-mediated cytotoxicity assays and to discriminate the effect of newly developed drugs on normal and cancer cells [57].

In another study, pMMA nanoparticles loaded with PTX were coated with GSH-CH and CH of different MWs, and their cytotoxic activity, mucoadhesiveness and release profiles were evaluated [58]. The resulting NPs were monodisperse and spherical in shape with a particle size ranging from 130 to 250 nm. GSH-CH films showed a higher mucoadhesion (MW dependent) than CH-coated NPs, and released 75% of PTX within 10 days. Additionally, NPs were very toxic for NIH 3T3 and T47D cells (breast carcinoma cells) and showed no toxicity for the HT29 and CaCo-2 (colon) cells. Likewise, coating had no effect on the cytotoxic activity, but GSH-CH coated NPs improved the residence time, and enhanced the release of PTX by providing a high mucoadhesiveness [58]. Furthermore, Lian and collaborators encapsulated PTX on nanomicelles of NAC-CH-vitamin E succinate improving its intestinal absorption to 4.5-fold in comparison to the free PTX. This system was also able to exhibit a sustained release profile up to 168h at a pH of 6.8. Moreover, the $AUC_{o\text{-}t}$ of PTX was clearly higher in NAC-CH-vitamin E succinate nanomicelles as compared to the free PTX and CH-vitamin E nanomicelles [59]. In addition, the cellular uptake and cytotoxicity of PTX against the A549 cells was enhanced when encapsulated in NPs composed of PLA-PCL-TPGS and TCH [60].

Saremi and collaborators produced NPs composed of a pMMA core surrounded by a shell of GSH-CH to enhance the bioavailability of DTX. The half-life of DTX-loaded NPs was ~ 9 times longer than those of free DTX and showed a 10-fold bioavailability enhancement. This nanoparticulate system decreased the transepithelial electrical resistance of the Caco-2 cell monolayer by opening the tight junctions [61]. In another study, NPs had a high cytotoxic effect in CaCo-2 and MCF-7 cell lines after 72 h and hence, were proposed as carriers for hydrophobic drugs [62].

Furthermore, the permeation and presence of DTX in the cells was increased by 9.6-fold and 13-fold, respectively due to encapsulation on folate-grafted TCH nanoliposomes. The resulting IC_{50} was 200-fold lower as compared to the non-encapsulated DTX on MD-MD-231 breast cancer cells [63].

On the other hand, NPs composed of methyl-β-cyclodextrin and poly (isobutylcyanoacrylate) coated with TCH (MW, 20 kDa) contained DTX improving the intestinal permeation due to a high mucoadhesion. Moreover, the DTX release in simulated intestinal fluid having pancreatin was 60% after 24h and reached a complete release after 48h [64].

Kim and collaborators developed a system for controlling DOX release using an AC magnetic field. The system was based on ferromagnetic disks cross-linked on their surface with DOX-TCH. The release of DOX was ~45% after 90 minutes, but after the application of a magnetic field for 10 minutes allowed for an additional 6% release. Consequently, these disks could be used for on-demand magneto-mechanically induced release of drug molecules [65].

Nanometric Systems

Martin and collaborators synthetized NPs with unmodified and TGA-conjugated chitosan at a pH of 4.0 and 5.0 and evaluated their uptake by CaCo-2 cells. They found that NPs having a diameter between 100 and200 nm had a low toxicity, and the TR was 5-fold higher with TCH than that of CH alone. TR of cross-linked TCH NPs was lower than that of the non-crosslinked product due to the low number of free thiol groups. Furthermore, NPs developed at pH of 4.0 showed promising results serving as a tool for the oral delivery of therapeutic genes [66].

Similarity, Yousefpour and collaborators synthesized NPs of GSH-CH and CH having different MWs by cross-linking with TPP. They observed that the inclusion of GSH neither modify the hydrodynamic diameter of the NPs, nor their surface charge. Additionally, a low MW polymer rendered a product with a higher thiolation degree due a major accessibility of the thiolating agents. Further, the mucoadhesiveness of NPs made of GSH-CH was not altered at a pH of 1.5, 3.5 and 5.5, showing potential as nanoparticulated oral drug delivery systems [67]. Moreover, the oxidative stability of GSH can be enhanced by immobilization on NPs and conjugates of GSH-CH. Further, NPs showed a 96% retention of thiol groups as compared to conjugates that only retained 55% of thiol groups [68].

Anitha and collaborators fabricated NPs with TCH loaded with 5-fluorouracil and curcumin, having an EE of 46.8% and 85.5%, respectively. The combination of these ACDs within the NPs enhanced the cytotoxic effect ranging from 2.5 to 3-fold on colon cancer (HT29) cells and improved their bioavailability. Furthermore, drug release was higher at a pH of 4.5 than 7.4 for both drugs [69].

NPs of TGA-CH cross-linked with TPP showed a cell viability larger than 98% at concentrations of 0.2-2.0 mg/mL on cancer and normal cells indicating a good biocompatibility. The particle size ranged from 80 to 100 nm and NPs were stable showing a zeta potential of +43.69. Furthermore, these systems could be used for drug and gene delivery applications due their excellent internalization [70].

Moreover, Gao and collaborators created a pH/redox responsive NPs of thiolated carboxymethyl chitosan for the encapsulation of MTX. NPs were spherical and stable in suspension at a pH of 7.4. The MTX release was around 19% at a pH of 7.4 containing 10 μM GSH, and 93% at a pH of 5.0 having a 20 mM GSH. The cytotoxicity of NPs reached 90% on HeLa cells [71]. Likewise, Alamdarnejad and collaborators grafted the TCH derivative onto cyclodextrin to form NPs that encapsulated albendazol,-a hydrophobic model drug (antiparasitic and anticancer drug).Their results indicated that the NPs were suitable to carry albendazol showing a high drug entrapment and slow release profile [72].

In another study, NPs of NAC-CH and NAP-CH were developed to entrap DOX and ASOND (antisense oligonucleotide), separately. The thiolated NPs released 22.8% of the drug and ASOND showing a higher stability, mucoadhesiveness and suppressed the EGFR gene expression in T47D (breast cancer) cells [25].

Further, Saboktakin and collaborators developed NPs of TCH-dextran sulfate employing the emulsion-solvent evaporation method. The type of polymer used in the formulation influenced size distribution, entrapment efficiency and release characteristics. NPs were able to encapsulate letrozole and showed a sustained release of letrozole representing an useful tool for targeting drug release at the absorption site [73].

On the other hand, Wang and collaborators could stabilize gold nanorods by using MAA-CH conjugates and demonstrated that TCH could replace cetyltrimethylammonium bromide, -a common agent used for the production of gold nanorods- that, unfortunately, enhanced its toxicity in cell-related studies. The new nanorods were much biocompatible and showed a better internalization due to the functionalization with folic acid on HT-29 cancer cells. These nanorods showed promising photothermal nano-absorption ability for ablation of cancer cells under the NIR laser exposure [74].

Microparticulated Systems

Pengpong and collaborators developed microparticles via electrospray ionization with TPP. These microparticles were composed of a new TCH named as homocysteine-p-coumaric acid-chitosan. These particles showed an 80% piperine encapsulation efficiency enhancing the oral bioavailability of curcumin once they were co-administered. TCH microparticles showed a higher drug release and mucoadhesiveness at a pH of 1.2, 4.0 and 6.4 than CH alone. Further, this new carrier was created for loading hydrophobic compounds by π-π interactions, which is different from other TCH derivatives [76].

RECOMMENDATIONS FOR IN-VITRO TCH TESTING IN CANCER CELL LINES

The cancer cell lines are the most widely used experimental models in cancer research due to their reproducible results that can be extrapolated to *in-vivo* models. Nevertheless, strict culture

conditions should be maintained to avoid genome instability, cross-contamination, and infections that might cause the cell line to express different characteristics from the primary tumor where they were isolated. This could lead to differences with the *in-vivo* test results. Therefore, it is necessary to control the number of passages, frozen cells, and regulate the cell population rate by limiting the conditions of the culture. Further, it is essential to conduct a DNA fingerprinting to ensure that the cell lines are identical to the tumor they were isolated from [76].

CONCLUSIONS

TCHs-based DDS have shown to be a magnificent tool for cancer targeting due to the IC_{50} decreased as compared to traditional ACD. These DDS exhibit a controlled and pH-dependent release showing a lower dosing scheme and wider frequency of administration. Moreover, these systems are capable to assess the administration of ACD by nasal and oral routes enhancing patient compliance. Furthermore, the production process of TCH is relatively easy and these derivatives conserve the key outstanding characteristics of CH such as biodegradability and biocompatibility.

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