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Ciprofloxacin chitosan conjugate: combined antibacterial effect and low toxicity

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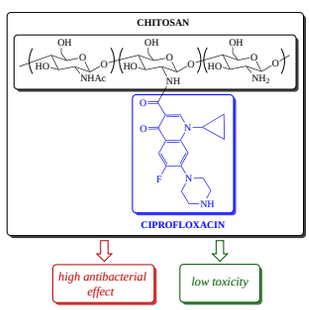
Ultrasound-mediated reaction of chitosan with ciprofloxacin in the presence of *N*,*N*′-dicyclohexylcarbodiimide in water gives the chitosan–ciprofloxacin conjugates of carboxamide type. The conjugates show a mediate antibacterial effect and reduced cytotoxicity.



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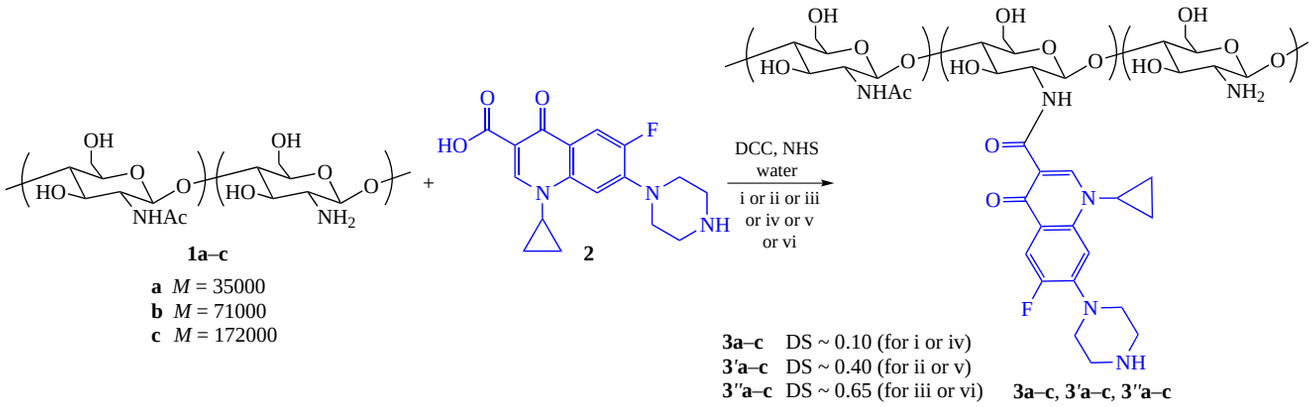
Fluoroquinolones are one of the most powerful antibiotics and are often the last resort that can save patient's life with a complicated course of an infectious disease and severe septic conditions.[1] However, the use of fluoroquinolones is associated with a number of limitations, the main of which are gradual formation of bacterial resistance to fluoroquinolones and their toxic effects.[2] These problems can be overcome by conjugating fluoroquinolones with a polymer matrix. In particular, our group recently demonstrated that the conjugation of the azide antibacterial compound, 1-azido-3-chloropropan-2-ol, with chitosan led to dramatic decrease in toxicity without losing the antibacterial effect.[3]

Chitosan is a seminatural biocompatible, biodegradable polymer characterized by the absence of carcinogenicity and toxicity.[4–7] An important advantage of chitosan in comparison with other natural polymers is the convenience of its chemical modification due to the presence of the primary amino group.[8,9] Fluoroquinolones contain carboxy group in the molecule and, therefore, can be conjugated with NH_2 group of chitosan *via* covalent amide bond. To the best of our knowledge, such conjugates of fluoroquinolone with chitosan are not documented. Within the framework of the current study, we herein carried out the synthesis of the proposed conjugates followed by assessment of their antibacterial activity and toxicity.

For formation of the amide bond by reaction of carboxylic acid with primary amines, usually commercially available cheap *N*,*N*′-dicyclohexylcarbodiimide (DCC) is used, frequently in the presence of *N*-hydroxysuccinimide (NHS). However, in chitosan chemistry, application of DCC is limited by insolubility and rapid hydrolysis in water that significantly reduces the yield and substitution degree (SD) in the resulting product.[10] In order to carry out the reaction in a homogeneous mode, water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is preferred,[11] however, this reagent is much more expensive. Recent studies demonstrated that ultrasound stimulates a number of heterogeneous reactions with chitosan leading to dramatic decrease in the reaction time and the required amount of reactants.[12–20] Being inspired by these findings, we intended to perform the heterogeneous DCC-mediated conjugation of ciprofloxacin as a 'fluoroquinolone gold standard' and chitosan under ultrasonic treatment.

Optimization of the conditions for the DCC-mediated reaction of chitosan and ciprofloxacin in terms of the frequency and the output power of the applied ultrasound revealed that the optimum acoustic parameters lie in the range of 90–110 kHz and 270–290 W. Under these conditions, the reaction is completed in 20 min. To confirm the effect of ultrasound, DCC-mediated reaction of chitosan with ciprofloxacin (Scheme 1) was studied both under ultrasonic treatment and under control conditions.

We evaluated the influence of the required excess of ciprofloxacin to achieve the same degree of substitution (0.10, 0.40 and 0.65) without and with ultrasound. In all cases, the reactions were performed at pH 3 and 50 °C, the prolongation having been 5 h (without ultrasound) or 20 min (with ultrasonic irradiation at 100 kHz, 280 W). For example, the ultrasound-free reaction at the chitosan/ciprofloxacin molar ratio 1 : 6 gives product with the degrees of substitution of 0.65, while to reach the same degree of substitution under ultrasonic conditions, this parameter may be diminished to 1 : 3.5. Thus, ultrasound dramatically reduces both required excess of



Scheme 1 *Reagents and conditions*: i–iii, DCC and NHS (1 equiv. to **2** each), water, 50 °C, 5 h, **1/2** ratio (equiv./equiv.): for i, 1.5, for ii, 3.5, for iii, 6.0; iv–vi, DCC and NHS (1 equiv. to **2** each), water, 50 °C, sonication (100 kHz, 280 W), 20 min, **1/2** ratio (equiv./equiv.): for iv, 0.5, for v, 2.0, for vi, 3.5.

Table 1 Effect of ultrasonic irradiation on the synthesis of ciprofloxacin–chitosan conjugates.

Substitution degree achieved	Control conditions[a]		Ultrasonic conditions[a]	
	molar ratio	reaction time	molar ratio	reaction time
0.10	1.5	5 h	0.5	20 min
0.40	3.5	5 h	2	20 min
0.65	6	5 h	3.5	20 min

[a] For all cases, T = 50 °C and pH 3.

ciprofloxacin and the reaction time (Table 1). The promoting effects of ultrasound is usually explained by cavitation chemistry the main postulates of which are carefully and in detail reviewed elsewhere.[21]

The resultant polymers were characterized by ^{1}H NMR spectroscopy. The typical ^{1}H NMR spectrum with signal assessment is presented in Figure 1. Substitution degree (SD) of the conjugates was calculated from ^{1}H NMR integral intensities according to the formula:

$$SD = I_{(H1')}, \text{ when } I_{(H1)} + I_{(H1')} + I_{(H1'')} = 1.$$

The code names of the synthesized polymers, their degree of substitution and molecular weights of the starting chitosan are given in Table 2. Thus, we prepared low- (0.10), moderate- (0.40) and high- (0.65) substituted polymers starting from chitosan of low (3.5×10^4), medium (7.1×10^4) and high (17.2×10^4) molecular weight. The molecular weight has no significant effect on the outcome of the reaction either under ultrasonic or control conditions.

The resultant polymers with a low degree of substitution are insoluble in water but soluble in 1% acetic acid or 1% HCl solution. Moreover, being dissolved in these acids, the polymers do not precipitate from the solution when its pH is adjusted to 7.0 by addition of sodium hydrocarbonate. This is probably due to the complexity of the formation of the system of interchain hydrogen bonds necessary for precipitation. This complexity, in turn, may be due to the steric bulkiness of the fluoroquinolone fragment introduced into the polymer chain. The moderate- and the high-substituted products are water-soluble.

The *in vitro* antibacterial activity of the synthesized polymers was evaluated using agar diffusion method (see Table 2). Diffusion of an antibacterial compound into the agar inhibits the bacterial growth, forming the circular inhibition zone around the site of application of the antibacterial compound. The diameter of the zone of inhibition (in mm) is directly proportional to the antibacterial activity of the test compound. The antibacterial activity of the prepared polymers was found to increase with the increase in their SD value. The antibacterial activity of the

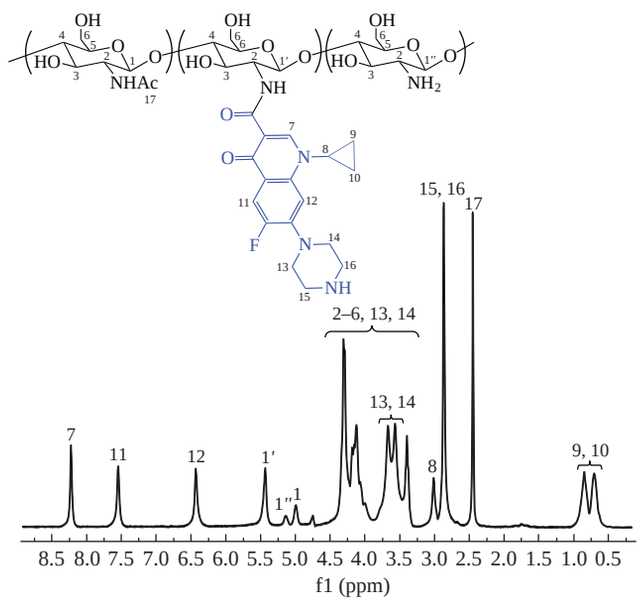


Figure 1 Typical ^{1}H NMR spectrum of ciprofloxacin–chitosan conjugate.

Table 2 Substitution degrees of the obtained ciprofloxacin–chitosan conjugates.

Sample	Molecular weight of the starting chitosan	Substitution degree	Inhibition zone/mm		Cell viability (%)
			E. coli	*S. aureus*	
3a	3.5×10^4	0.10	19.1±0.1	12.6±0.5	90
3b	7.1×10^4	0.10	19.2±0.4	12.9±0.5	89
3c	17.2×10^4	0.10	18.7±0.2	12.3±0.2	87
3′a	3.5×10^4	0.40	30.4±0.4	25.7±0.2	90
3′b	7.1×10^4	0.40	32.5±0.2	26.2±0.2	90
3′c	17.2×10^4	0.40	29.6±0.2	25.1±0.1	87
3″a	3.5×10^4	0.65	41.7±0.2	36.4±0.2	89
3″b	7.1×10^4	0.65	43.5±0.3	38.1±0.5	89
3″c	17.2×10^4	0.65	41.0±0.1	35.9±0.3	85
Ciprofloxacin	–	–	43.5±0.4	38.3±0.2	50

conjugates is slightly dependent on their molecular weight, however, the derivatives of chitosan of medium molecular weight are somewhat more active than their low and high molecular weight analogues. The most active antibacterial compound proved to be **3″b**. The minimum inhibitory concentrations (MIC, μg ml^{-1}) of **3″** are 0.11 (*E. coli*) and 2.08 (*S. aureus*). The MIC for ciprofloxacin are 0.10 (*E. coli*) and 2.10 (*S. aureus*). Thus, the *in vitro* antibacterial effect of **3″b** is comparable with that of ciprofloxacin.

The cytotoxicity of the most active polymer **3″b** toward mammalian cells was studied *in vitro* in HEK-293 cell culture. Even when using concentration of **3″b** 200 μg ml^{-1}, which is significantly higher than the MIC, the cell viability was *ca*. 90%. The same concentration of ciprofloxacin causes 50% cell viability (see Table 2). Hence, **3″b** is significantly less toxic than ciprofloxacin.

In summary, the first conjugates of ciprofloxacin with chitosan were prepared using DCC as the convenient amidation reagent. We recognized that the conjugation of ciprofloxacin with chitosan significantly reduces its toxicity without losing its antibacterial effect. Moreover, we identified conjugate-leader **3″b**, which is interesting for further *in vivo* studies.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2022.11.022.

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