

## Characterization of chitosan hydrochloride–mucin interaction by means of viscosimetric and turbidimetric measurements

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### Abstract

In the present work the interaction between chitosan hydrochloride (HCS) and two different types of mucin – one obtained from bovine submaxillary glands and the other from porcine stomach – was investigated. Two hydration media were tested: distilled water and 0.1 M HCl. Intrinsic viscosity, which provides information about polymeric chain conformation, was assessed in both media for HCS and bovine submaxillary mucin. Changes in the specific viscosity of HCS–mucin mixtures were observed as a function of the polymer:mucin weight ratio. The formation of interaction products was indicated by a minimum in the specific viscosity. Such a minimum occurred at different polymer:mucin weight ratios depending on the hydration medium and mucin type. This suggested a different stoichiometry of interaction. Turbidimetric measurements were also effected in order to evidentiate the eventual precipitation of the polymer–mucin interaction products. While in distilled water the precipitation of the interaction product did occur, in acidic medium, although a minimum in specific viscosity was observed, no precipitate was formed. The two techniques employed, viscosimetric and turbidimetric, allowed us to investigate for both mucins the influence of hydration medium on the formation of the HCS–mucin interaction products and to conclude that a slightly acid–neutral pH favours the interaction between HCS and mucins. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Chitosan hydrochloride; Polymer–mucin interaction product; Intrinsic viscosity; Specific viscosity; Turbidity

### 1. Introduction

Chitosan ([1–4]2-amino-2-desoxy- $\beta$ -D-glucan) is a natural polycationic polymer which has valuable properties as a biomaterial (Felt et al., 1998). Standard grades of chitosan require the addition of acid to solubilize; in fact chitosan is a weak base and a certain amount of acid is required to transform the glucosamine units into the positively charged, water soluble form. Acetic acid and hydrochloric acid are the most commonly employed, but other acids (lactic acid, glutamic acid, etc.) are successfully used.

Chitosan biocompatibility, biodegradability and low toxicity together with the cationic character and the potential reactive groups make it an attractive biopolymer for many biomedical and pharmaceutical applications. For instance, chitosan has demonstrated that it enhances the absorption of peptide drugs in vitro (Illum et al., 1994; Kotze et al., 1999; Witschi and Mrsny, 1999) and has also shown some pharmacological activities as an antacid,

antiulcer and a hypocholesterolemic agent (Felt et al., 1998).

As a pharmaceutical excipient, chitosan has been used in a variety of conventional formulations (such as powders, granules, direct compressed tablets, wet-granulated tablets, emulsions and gels) and in controlled release microparticles (Illum, 1998; Remunan-Lopez et al., 1998a; He et al., 1999). In view of its employment in controlled drug delivery, interpolymer complexation of chitosan with sodium alginate, sodium polyacrylate, acacia and pectin has been investigated (Takahashi et al., 1990; Meshali and Gabr, 1993).

Chitosan has also demonstrated that it possesses mucoadhesive properties due to the formation, depending on environmental pH, of either secondary chemical bonds such as hydrogen bonds or ionic interactions between the positively charged amino groups of chitosan and the negatively charged sialic acid residues of mucus glycoproteins or mucins (Lehr et al., 1992; Rilloso and Buckton, 1995). In particular, some authors (Fiebrig et al., 1994; Deacon et al., 1999) pointed out the formation of a chitosan–pig gastric mucin complex by means of sedimentation experiments. A positive rheological synergism

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was observed by other authors when chitosan solutions, prepared in pH 5.5 acetate buffer and in 0.1 M HCl, were mixed with porcine gastric mucin: the mixtures with mucin showed a viscosity greater than the sum of the polymer and mucin viscosities (Hassan and Gallo, 1990; Ferrari et al., 1997a). This indicated the occurrence of mucoadhesive interactions between the two macromolecular species.

Recently, various mucoadhesive formulations based on chitosan have been developed. They include tablets (Takayama et al., 1990; Miyakazi et al., 1995), films (Lehr et al., 1992; Remunan-Lopez et al., 1998b), gels (Needleman and Smales, 1995; Valenta et al., 1998), liposomes (Henriksen et al., 1996; Takeuchi et al., 1996; Uchegbu et al., 1998), microparticles (Ferrari et al., 1997b; Genta et al., 1997; Shah et al., 1997).

Given the mucoadhesive properties of chitosan and its capability of interacting with anionic polymers to form complexes, it was interesting to investigate the interaction between the water soluble chitosan.HCl (HCS) and mucins. In particular, the influence of the polymer hydration medium and the mucin type on the formation of the polymer–mucin interaction products was investigated. Two different hydration media, distilled water and 0.1 M HCl, in which HCS had shown different rheological properties (Ferrari et al., 1997a), were used. Two commercial mucins of differing origin and solubility were employed.

The influence of the hydration medium on the intrinsic viscosity of HCS and mucin was first assessed in order to obtain information on the polymeric chain conformation in the two media (Kurata and Tsunashima, 1989).

HCS solutions, prepared in distilled water and in 0.1 M HCl, were then mixed with increasing amounts of each and every mucin. The formation of polymer–mucin interaction products was determined on the basis of the changes in specific viscosity of the mixtures as a function of polymer:mucin weight ratio. Turbidimetric measurements were also effected in order to evidentialize the eventual precipitation of interaction products.

## 2. Materials and methods

### 2.1. Materials

Chitosan.HCl (high viscosity-grade, deacetylation degree=84%) (HCS) (Seacure® CL 313, Pronova Biopolymer a.s., Drammen, Norway) was used as test material. Two commercial mucins were used as biological substrate: purified mucin from bovine submaxillary glands (type I) (BSMG) and partially purified mucin from pig stomach (type III) (PS), both purchased from Sigma Chimica (Milan, Italy).

It is known that the use of both commercial and freshly prepared mucin samples involves advantages and drawbacks (Rossi et al., 1995). The use of commercial mucins

in the present paper is justified by the lower batch-to-batch variability shown by commercial samples with respect to those freshly prepared.

### 2.2. Sample preparation

Chitosan.HCl (HCS) and BSMG and PS mucins were hydrated in distilled water and in 0.1 M HCl by gentle stirring at room temperature. HCS and BSMG mucin were soluble in both media, whereas PS mucin was partially soluble and therefore produced dispersions.

The concentrations of HCS solutions were: 0.008, 0.006, 0.004 and 0.002% (w/v) in distilled water and 0.08, 0.06, 0.04 and 0.02% (w/v) in 0.1 M HCl. The pH of HCS solutions was 4.6 in distilled water and 1 in acidic medium, respectively.

The concentrations of BSMG mucin solutions were: 0.06, 0.04, 0.02 and 0.01% (w/v) in distilled water and 0.16, 0.12, 0.08 and 0.04% (w/v) in 0.1 M HCl. The pH of BSMG mucin solutions was 6.8 in distilled water and 1 in acidic medium, respectively.

The concentrations of PS mucin dispersions were: 0.16, 0.12, 0.08 and 0.04% (w/v) in both media. The pH of PS mucin dispersions ranged from 4.7 to 5.1 in distilled water and was 1 in acidic medium.

The HCS solutions and mucin solutions/dispersions were tested 5 h after preparation. The concentrations of both HCS and mucins were properly chosen in order to obtain relative viscosity values in the range 1.1–1.5 suitable for the calculation of intrinsic viscosity (Martin, 1993). For HCS and BSMG mucin solutions different concentrations were used in the two media since both the polymers showed higher viscosities in distilled water than in 0.1 M HCl.

Mixtures containing a fixed amount of HCS and increasing mucin amounts (either BSMG or PS) were prepared: mucin solution/dispersions were added to already hydrated HCS. The mixtures were gently stirred for 5 h before testing.

Polymer:mucin weight ratios ranged from 1:0.5 to 1:20 and from 1:0.5 to 1:5 in distilled water and in 0.1 M HCl, respectively. Three different HCS concentrations were considered: 0.008, 0.004 and 0.002% (w/v) in distilled water and 0.08, 0.04 and 0.02% (w/v) in 0.1 M HCl.

### 2.3. Viscosity measurements

The specific viscosity ( $\eta_{sp}$ ) of all the samples prepared (HCS solutions, BSMG mucin solutions, PS mucin dispersions and polymer–mucin mixtures) was determined at 37°C by means of a Cannon-Fenske capillary-tube viscosimeter (S50) according to the following equation:

$$\eta_{sp} = (\eta/\eta_0) - 1$$

where  $\eta$ =viscosity of the sample,  $\eta_0$ =viscosity of the

hydration medium. Three replicates were obtained for each sample and three measurements were effected on each replicate.

The HCS–BSMG mucin mixtures prepared in distilled water were filtered under vacuum (SSWP membrane filter,  $\varnothing=3$  mm, Millipore, Milan, Italy) before viscosity measurements in order to remove the eventual precipitate due to the polymer–mucin interaction. Filtration was continued until the filtrates showed constant specific viscosity values.

For HCS and BSMG mucin reduced viscosity ( $\eta_{sp}/c$ ) was also calculated as the ratio between  $\eta_{sp}$  and the polymer concentration. The  $\eta_{sp}/c$  values were plotted against the corresponding polymer concentrations. Intrinsic viscosity,  $[\eta]$ , was calculated by extrapolation to zero concentration (Kurata and Tsunashima, 1989). Three straight lines, corresponding to the three replicates, were obtained in each medium and the intrinsic viscosity was calculated as the mean of the three extrapolated values.

#### 2.4. Turbidimetric measurements

Turbidimetric measurements were effected by means of a Spectracomp 602 spectrophotometer (Advanced Products, Milan, Italy) at 650 nm.

The precipitation of the interaction product between the polymer and BSMG mucin in distilled water was assessed by visual observation. To ascertain what had been visually observed, turbidity was measured for the HCS–BSMG mixtures prepared in distilled water at 0.008% (w/v) HCS concentration.

For the HCS–PS mucin mixtures, the precipitation of the interaction product could not be visually assessed, since PS mucin itself does not produce clear solutions either in distilled water nor in 0.1 M HCl. Turbidity of the HCS–PS mucin mixtures prepared in distilled water and in 0.1 M HCl and containing HCS at 0.008 and 0.08% (w/v), respectively, was compared with that of PS mucin dispersions containing the same mucin amount as the mixtures.

### 3. Results and discussion

#### 3.1. Viscosity measurements

##### 3.1.1. HCS solutions

HCS shows different values of intrinsic viscosity in distilled water ( $[\eta]=59.9\pm0.9$  dl/g) and in 0.1 M HCl ( $[\eta]=7.5\pm0.2$  dl/g). Since intrinsic viscosity is an indirect measure of the size and shape of polymeric chains (Kurata and Tsunashima, 1989), the comparison of  $[\eta]$  values obtained for the same polymer hydrated in different media provides information about the changes in the polymer conformation occurring in those media. The lower  $[\eta]$  value observed in 0.1 M HCl indicates, therefore, a different polymer conformation with respect to that occurring in distilled water, in particular a contraction of the

polymeric coil. This is probably due to a shielding effect of the excess of  $\text{Cl}^-$  ions, which, having an opposite charge, decrease the intramolecular repulsion of the polymeric chains leading to a tighter conformation and, consequently, to a lower intrinsic viscosity.

##### 3.1.2. BSMG mucin solutions

As already observed for HCS, BSMG mucin obtains a higher intrinsic viscosity value in distilled water ( $[\eta]=9.8\pm0.1$  dl/g) than in 0.1 M HCl ( $[\eta]=0.57\pm0.01$  dl/g). This again indicates that polymer macromolecules are characterized by different conformation in the two media. In distilled water, the partially ionized mucin chains repulse each other; this causes a more extended polymer conformation in distilled water than in acidic medium where mucin sialic acid residues are unionized.

##### 3.1.3. PS mucin dispersions

Since PS mucin was not completely soluble in both distilled water and in 0.1 M HCl, it was not possible to calculate for such mucin reduced viscosity and, then, intrinsic viscosity. Therefore, the comparison of mucin behaviour in the two media was effected on the basis of  $\eta_{sp}$  values.

In Fig. 1  $\eta_{sp}$  values observed for PS mucin dispersions prepared at different concentrations in distilled water and in 0.1 M HCl are reported. It can be observed that, at least for the concentrations tested,  $\eta_{sp}$  was not affected by the hydration medium employed.

For PS mucin, characterized by a lower solubility than BSMG mucin, it is conceivable that the differences in viscosity expected in the two media are hindered by the mucin in suspension which plays a major role in determining sample viscosity.

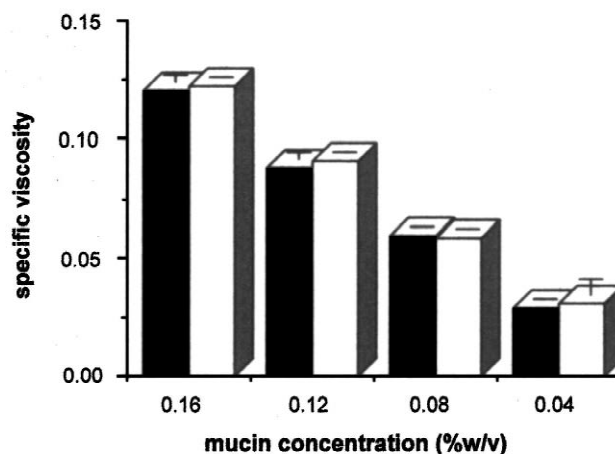


Fig. 1. Specific viscosity (mean values  $\pm$  S.D.;  $N=3$ ) observed for PS mucin dispersion prepared at different concentrations in distilled water and in 0.1 M HCl.

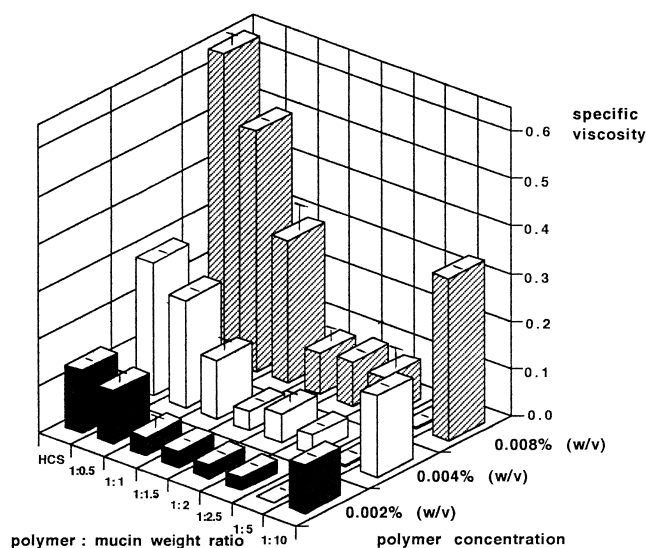


Fig. 2. Specific viscosity (measured after filtration) of HCS–BSMG mucin mixtures (mean values  $\pm$  S.D.;  $N=3$ ) prepared in distilled water as a function of polymer:mucin weight ratio and polymer concentration.

### 3.1.4. HCS–BSMG mixtures

#### 3.1.4.1. Distilled water

The addition of increasing BSMG mucin amounts to HCS hydrated in distilled water makes the HCS solution turbid; this is attributable to the formation and precipitation of an interaction product between the two macromolecular species.

In Fig. 2 specific viscosity values (measured after filtration) of HCS–BSMG mucin mixtures prepared in distilled water as a function of polymer:mucin weight ratio and of polymer concentration is reported. For all the three HCS concentrations, mixing with mucin produces a continuous decrease in the specific viscosity of the filtered solutions on decreasing polymer:mucin weight ratio. This is caused by the precipitation of the polymer–BSMG mucin interaction product. For a polymer:mucin weight ratio of 1:5, the viscosity of the filtered solution reaches a minimum almost equal to the viscosity of distilled water ( $\eta_{sp} \sim 0$ ), thus indicating an almost complete interaction between HCS and mucin. For a polymer:mucin weight ratio of 1:10, an increase in specific viscosity is observed, such an increase is conceivably due to the excess mucin

which has not interacted with the polymer. These results indicate that the stoichiometry of the interaction product is close to a 1:5 weight ratio.

The stoichiometry of the interaction product is confirmed through the quantification of the mucin excess in the mixtures prepared at the 1:10 polymer:mucin weight ratio. To quantify such an excess, the specific viscosity values observed for the 1:10 mixtures are substituted into the straight line equations  $\eta_{sp}/c$  vs.  $c$  used to calculate intrinsic viscosity. Since the slope and the intercept of such equations are known, the mucin concentration which corresponds to a given  $\eta_{sp}$  value can be calculated and this represents the mucin excess. From this value, the stoichiometry of the interaction product is calculated as described in Table 1. For the three HCS concentrations, the mucin involved in the interaction product is calculated by subtracting the mucin excess from the total mucin in the mixtures. The stoichiometry of the interaction product ranges from 1:4.9 to 1:5.7 and is in good agreement with the 1:5 stoichiometry determined on the basis of the changes in  $\eta_{sp}$  of the HCS–BSMG mucin mixtures (Fig. 2).

#### 3.1.4.2. 0.1 M HCl

In Fig. 3 specific viscosity of HCS–BSMG mucin mixtures prepared in 0.1 M HCl as a function of polymer:mucin weight ratio and of polymer concentration is reported.

When BSMG mucin is added to HCS hydrated in 0.1 M HCl, no precipitation occurred. For all the three HCS concentrations, only a slight decrease in specific viscosity, which reaches a minimum at a 1:1 weight ratio, is observed. This is probably due to the formation of an interaction product characterized by a lower affinity for the solvent and, then, by a lower viscosity. A further addition of mucin produces an increase in specific viscosity. This indicates that the stoichiometry of the interaction product is close to 1:1.

A lower mucin amount is required to form the interaction product in acidic medium than in distilled water.

The differences observed in the two media are conceivably due to the different ionization degree of chitosan D-glucosamine ( $pK_a=6.5$ ) and mucin sialic acid ( $pK_a=2.6$ ) residues in water and in 0.1 M HCl.

This result is also in line with the different chain

Table 1  
Stoichiometry of the interaction product<sup>a</sup>

HCS (%, w/v) (a)	Total mucin in the mixtures (%, w/v) (b)	Mucin excess (%, w/v) ( $\pm$ S.D.; $N=3$ ) (c)	Int. product stoichiometry (pol:muc weight ratio) [a/(b – c)]
0.008	0.080	0.0340 $\pm$ 0.0002	1:5.7
0.004	0.040	0.0173 $\pm$ 0.0002	1:5.6
0.002	0.020	0.0102 $\pm$ 0.0001	1:4.9

<sup>a</sup> Calculated on the basis of  $\eta_{sp}$  values observed after removal of the interaction product for the mixtures prepared at a 1:10 polymer:BSMG mucin weight ratio.

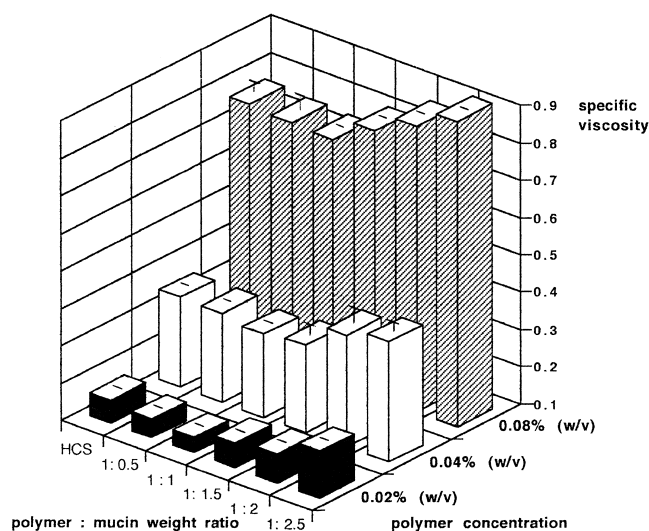


Fig. 3. Specific viscosity of HCS–BSMG mucin mixtures (mean values  $\pm$  S.D.;  $N=3$ ) prepared in 0.1 M HCl as a function of polymer:mucin weight ratio and polymer concentration.

conformation of both HCS and mucin in the two media, as indicated by intrinsic viscosity values. In fact, since both HCS and mucin chains have a tighter conformation in 0.1 M HCl, it is conceivable that they are less available to interact in acidic medium.

### 3.1.5. HCS–PS mixtures

#### 3.1.5.1. Distilled water

In Fig. 4 specific viscosity values of HCS–PS mucin mixtures prepared in distilled water as a function of polymer:mucin weight ratio and of polymer concentration are reported. For all the three HCS concentrations examined, a decrease in specific viscosity is observed on

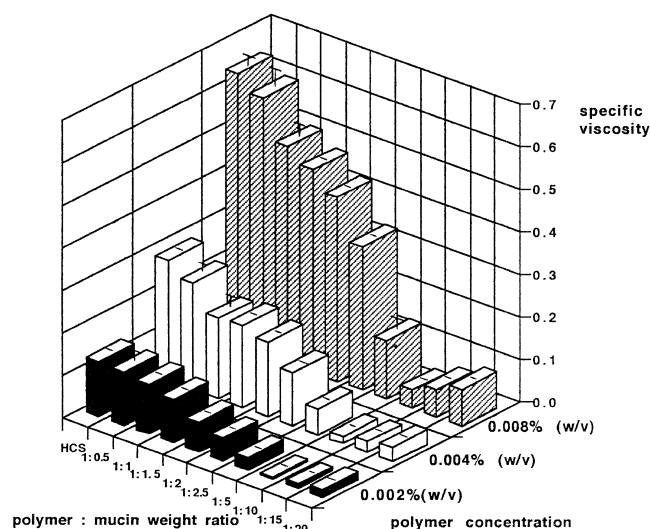


Fig. 4. Specific viscosity of HCS–PS mucin mixtures (mean values  $\pm$  S.D.;  $N=3$ ) prepared in distilled water as a function of polymer:mucin weight ratio and polymer concentration.

increasing mucin concentration up to a polymer:mucin weight ratio equal to 1:10. A further increase in mucin concentration causes an increase in specific viscosity. This indicates that the stoichiometry of the interaction product is close to 1:10. The specific viscosity at the 1:10 weight ratio is higher than 0 due to the fact that the interaction product was not removed by filtration from the samples. Filtration was not effected since it would have also removed the PS mucin that was not completely soluble in the two media.

#### 3.1.5.2. 0.1 M HCl

In Fig. 5 specific viscosity values of HCS–PS mucin mixtures prepared in 0.1 M HCl as a function of polymer:mucin weight ratio and of polymer concentration are reported.

In an acidic medium, the specific viscosity decreases on increasing mucin concentration. For all the three HCS concentrations examined, the minimum value of  $\eta_{sp}$  is reached for a polymer mucin weight ratio equal to 1:2. As already observed for BSMG mucin, a lower mucin amount is required in acidic medium than in distilled water to form the polymer–PS mucin interaction product.

### 3.2. Turbidimetric measurements

#### 3.2.1. HCS–BSMG mucin mixtures

In Fig. 6 turbidity values are given as a function of mucin concentration for the HCS–BSMG mucin mixtures prepared in distilled water at 0.008% (w/v) HCS concentration. Turbidity linearly increases on increasing mucin concentration up to 0.04% (w/v), which corresponds to a 1:5 polymer:mucin weight ratio. A further addition of mucin does not produce any change in mixture turbidity. This confirms the results obtained from viscosity

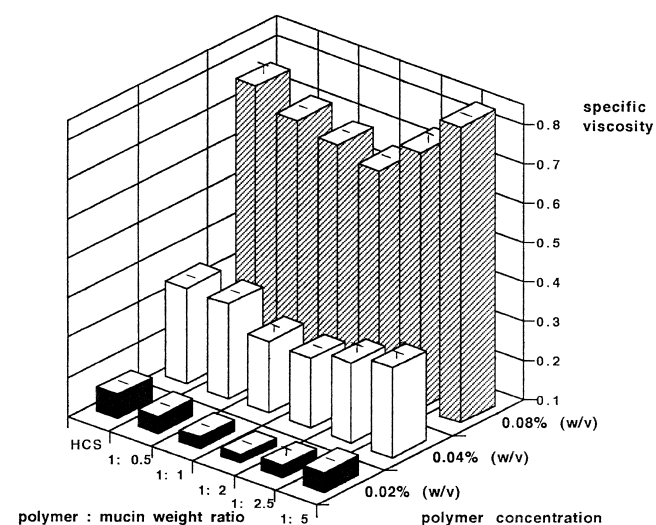


Fig. 5. Specific viscosity of HCS–PS mucin mixtures (mean values  $\pm$  S.D.;  $N=3$ ) prepared in 0.1 M HCl as a function of polymer:mucin weight ratio and polymer concentration.

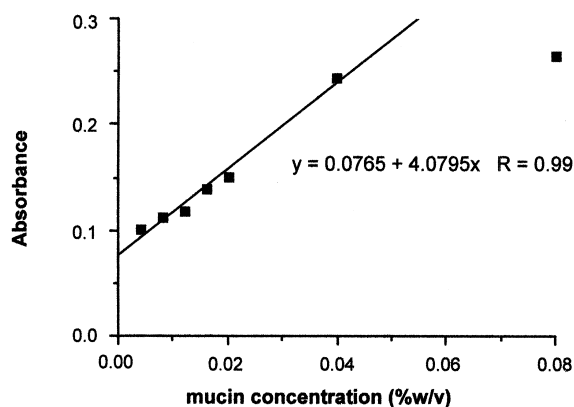


Fig. 6. Turbidity as a function of mucin concentration for HCS-BSMG mucin mixtures prepared in distilled water at 0.008% (w/v) HCS concentration.

measurements: the precipitation of the polymer-BSMG mucin interaction product is complete at a weight ratio close to 1:5.

### 3.2.2. HCS-PS mucin mixtures

In Fig. 7 turbidity values are given as a function of mucin concentration for the HCS-PS mucin mixtures and

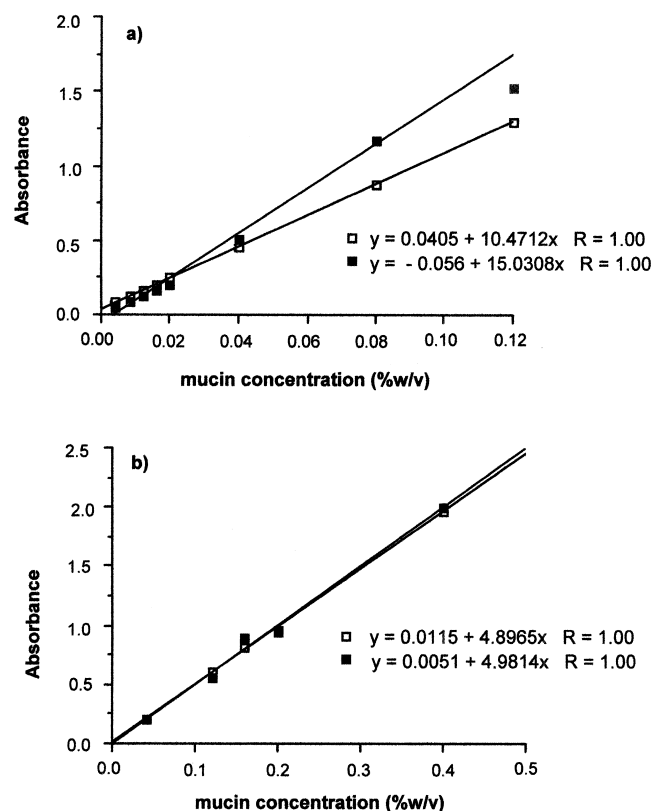


Fig. 7. Turbidity as a function of mucin concentration for PS mucin dispersions and HCS-PS mucin mixtures containing (a) 0.008% (w/v) of HCS in distilled water and (b) 0.08% (w/v) of HCS in 0.1 M HCl. □, Mucin dispersion; ■, polymer-mucin mixture.

for the corresponding PS dispersions prepared in (a) distilled water and (b) in acidic medium.

Turbidity versus concentration profiles observed for the mucin dispersions and for the polymer-mucin mixtures prepared in distilled water up to a 1:10 polymer:mucin ratio [mucin concentration=0.08% (w/v)] are well described by two straight lines characterized by different slopes (higher in the case of the mixtures) (Fig. 7a). The HCS-PS mixtures in distilled water show turbidity values higher than the corresponding mucin dispersions for mucin concentrations greater than 0.02% (w/v), which corresponds to a 1:2.5 polymer:mucin ratio.

For the mixture prepared at a weight ratio of 1:15 [mucin concentration=0.12% (w/v)], a lower turbidity than that expected on the basis of the straight line equation is obtained. This confirms that the addition of mucin beyond the 1:10 polymer:mucin weight ratio does not produce a further precipitation of the interaction product since, as previously demonstrated (Fig. 4), the polymer completely precipitates at the 1:10 polymer:mucin weight ratio. The increase in turbidity observed for 1:15 mixture is only due to the presence of mucin excess. Once again, the results obtained confirm that for a polymer:mucin weight ratio close to 1:10, the polymer precipitates completely as interaction product.

In acidic medium, no differences in turbidity between the mucin dispersions and the polymer-mucin mixtures are observed (Fig. 7b). This means that the interaction product does not precipitate.

## 4. Conclusion

The formation of the polymer-mucin interaction products is demonstrated by the occurrence of a minimum in the specific viscosity of the mixtures prepared at different polymer:mucin weight ratios. This result seems in disagreement with those found in previous papers (Hassan and Gallo, 1990; Ferrari et al., 1997a), where a synergic increase in viscosity was found when chitosan hydrated in 0.1 M HCl was mixed with mucin. This discrepancy is reasonably due to the different HCS concentrations and HCS:mucin weight ratios considered. HCS concentrations employed in the present work are markedly lower than those used in the previous studies. Work is in progress to clarify the influence of polymer concentration and polymer:mucin weight ratios on the formation of the polymer-mucin interaction product.

Chitosan.HCl interacts with both mucin types. The interaction products form at different polymer:mucin ratios depending on the mucin type and hydration medium. A higher mucin amount is necessary to form the interaction product with PS mucin than with BSMG mucin. This can be due to the fact that PS mucin is partially soluble in both hydration media employed and therefore a lower mucin amount is actually available for interaction. As for the

differences observed in the two media (occurrence of a precipitate and different stoichiometry of the interaction products), they are conceivably due to the different ionization degree of the two polymers in water and in 0.1 M HCl. Chitosan.HCl and BSMG mucin also show a different chain conformation in the two media. In fact, chitosan and mucin macromolecules are characterized by a more extended conformation in distilled water than in 0.1 M HCl, where a contraction of the polymeric coil occurs: this could prevent a deep interpenetration between polymer and mucin chains and therefore affect the chitosan interaction properties.

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