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Insight into the Heparin–Toluidine Blue (C.I. Basic Blue 17) interaction

Lucio D'Ilario*, Iolanda Francolini, Andrea Martinelli, Antonella Piozzi

Chemistry Department, University of Roma "La Sapienza", PO Box 34, Roma 62, Roma, Italy

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ABSTRACT

Heparin–Toluidine Blue (C.I. Basic Blue 17) interaction was studied using UV–vis spectroscopic analysis of the titration of the functional groups present on the polyanion chain. The study took into consideration both the pH and relative amount of the dye as a function of the Heparin repeat unit number; formation of a dye/polysaccharide aggregate was studied under the same conditions and structural models of the two types of aggregates were proposed. As a consequence of such an investigation a new Heparin determination methodology was developed.

1. Introduction

Heparin (Hep) [1,2] is a linear natural polysaccharide whose importance has grown enormously because of its very large clinical use as an anticoagulant agent. Moreover, nowadays polymeric materials which could be employed in biomedical applications such as prostheses and implants are normally made blood-compatible by physical and/or chemical Hep immobilization.

Hep is a glycosaminoglycan polymer whose approximate molecular formula is IdoA(2S)–GlcNS(6S)–GlcA–GlcNS(6S), the uronic acid within the disaccharidic being either the α-L-iduronic acid (IdoA) or the β-L-glucuronic acid (GlcA) (Fig. 1).

The biological activity of Heparin is due to the presence of the following functional groups: two carboxylic, three *O*-sulfate and two *N*-sulfate groups. The natural polysaccharide origin makes variable the molecular weight, ranging between 3.103 and 3.104, and the *n* and *m* indices. However, in the research work the commercial Hep used is generally one of a low molecular weight, obtained from porcine intestinal mucosa or from bovine lung, so limiting the molecular heterogeneity of the polysaccharide.

The Hep determination may be accomplished in a number of ways: biologically [APTT test [3] and antithrombin-III method [4]], radiochemically [isotopic substitution with 35S] [5,6], chemically [elemental S analysis] [7], colorimetrically and fluorimetrically [8–12], HPSEC [13,14], by agarose-gel electrophoresis [15,16] and by HPLC of the unsaturated disaccharides after treatment with specific lyases [17–20].

Precision and simplicity are of course the main prerequisite for any analytical determination. The colorimetric assay of Heparin by means of metachromatic dyes such as Acridine Orange and Toluidine Blue (C.I. Basic Blue 17; TB) is undoubtedly easy and quick and it has been adopted since 1941 when MacIntosh published in Biochem. J. his method of standardisation of Heparin preparations [8] (Fig. 2).

Smith and colleagues in 1980 employed such a method for the determination of Heparin content in immobilized Heparin preparations [12]. This analysis makes use of the color change of the dye, accompanied by a shift to a shorter wavelength of the absorbance, in the presence of charged polymeric species, e.g. tissue such as cartilage, and, in general, polyelectrolites. Such a phenomenon, widely used by the histologists, is known after Ehrlich in 1877 as *metachromasia*, and is attributed to the so-called dye polymerization on the polyanionic substrate.

Although a very large number of papers are present in the literature concerning both the dye interaction with polyelectrolites and its own physico-chemical properties, the phenomenon of metachromasia has not been completely elucidated, making partially uncertain any analytical determination based on it. Only recently an effort aimed at elucidating the aggregation and structural aspects of the Toluidine Blue dye metachromatic behaviour by itself was made in our laboratory [21] by obtaining the molar spectra of the different aggregation species and a structural model of the dimeric form of TB as well as by studying its supramolecular

* Corresponding author. Tel./fax: +39 06 4991 3740.
E-mail address: lucio.dilario@uniroma1.it (L. D'Ilario).

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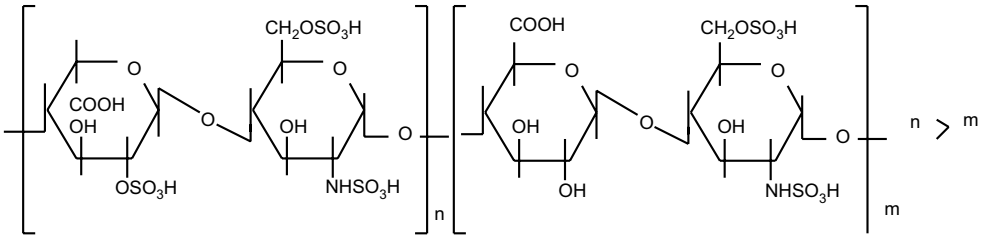


Fig. 1. Heparin, approximate molecular formula.

organization in the solid amorphous phases [22]. This may help the understanding of the dye–polyelectrolite interaction optical phenomena.

The very large use of such a methodology and some of its peculiarities has then directed us to perform more detailed investigations. In the present paper we discuss some of the crucial points regarding the TB/Hep interaction and show how, for analytical purposes, the Hep determination may be accomplished in a simpler, faster and more reliable way.

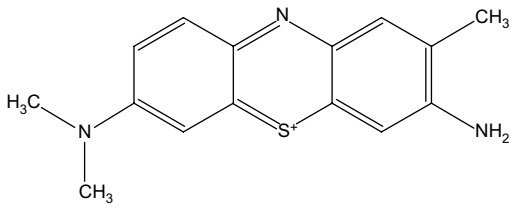
2. Materials and methods

Heparin from porcine intestinal mucosa, in its sodium salt form, was purchased from Fluka. The molecular weight was in the 4000–6000 Dalton range and its biological activity was ≈140 units/mg. The Toluidine Blue used, CA Index Name *Phenothiazin-5-ium, 3-amino-7-(dimethylamino)-2-methyl-, chloride,* was in the $ZnCl_2$ complex dimeric form, when purchased as Standard Fluka for microscopy, and in its monomeric form when obtained from Sigma (purity degree 90%). It was subjected to the purification procedure of Pal and Schubert [23] prior to use. Briefly, after this treatment no more differences were found in the dyes coming from the two different firms. The other chemicals employed were of standard reagent grade.

Our experiments were carried out on aqueous NaCl (0.2% w/v)solutions, acidified at pH = 2.06 by HCl addition.

The spectrophotometric measurements were accomplished by means of an HP 8452A Diode Array instrument operating with a resolution of ±2 nm and a photometric accuracy of ±0.005 AU. A Cary 5E UV–VIS–NIR spectrophotometer with a wavelength accuracy of ±0.1 nm and a reproducibility of ±0.3 nm was also employed at a scan speed of 600 nm/min. The temperature was always maintained at 25 °C. All the measurements were carried out by using a so-called "kinetic method" based on a titration apparatus working in continuum and shown in Fig. 3.

The method consisted of a Heparin solution photometric titration by the continuum addition of a known concentration dye solution. The measurement cell was an Hellma flux cell with a volume of 80 μl and an optical path length of 10 mm. The working rate of the peristaltic pump feeding the reaction cell with the dye was two orders of magnitude lower than that feeding the measurement cell in order to allow the stationary state to be reached within the reaction cell before the spectrophotometric reading. The TB volumes added to the Heparin solution were always negligible compared to the latter, so that the volume variation could not be taken into account.



Toluidine Blue
(3-amino-7-(dimethylamino)-2-methylphenothiazin-5-ium)

Fig. 2. Toluidine Blue, molecular formula.

3. Results and discussion

In our previous work [21] on TB we hypothesised, on the basis of the exciton theory and of the Davidov splitting, a model spectrum of the dye due to the overlapping of the bands of 6 aggregate species and we could assign the characteristic parameters for each of them by assuming gaussian shapes of the peaks. Particular care was taken in obtaining the monomer spectrum, whose features, however, were found not perfectly represented by only one gaussian curve initially assigned in order to minimize the number of the fitting parameters. In fact the sensibly asymmetrical shape of the experimental spectrum of the monomeric species could be better represented by a couple of gaussian curves, which, however, would have increased the number of the degrees of freedom. By confining our attention just to the first three types of aggregates (monomer, dimer and trimer) the above-mentioned study showed that they could be represented, respectively, by one gaussian centered at 610 nm for the monomer, two gaussians centered at 580 nm and 640 nm for the dimer and one gaussian centered at 550 nm for the trimer.

Heparin is a high negative charge density natural polysaccharide with anticoagulant properties. It prevents the clotting of blood and the formation of thrombi constituted of fibrin clots and blood cells. Its importance has sensibly grown with the diffusion of more traditional and newer biomedical materials for the prosthetic applications and for the regenerative medicine and tissue engineering, on which Heparin is often bound to improve material hemocompatibility. In these kinds of applications the need of a reliable Hep determination method that is easy and fast is essential. The colorimetric methods employing TB are those of our

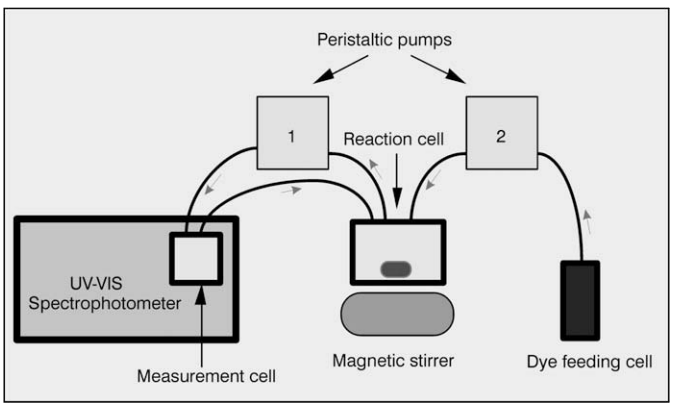


Fig. 3. Spectrophotometric titration apparatus.

interest mainly because of their simplicity. However, the dye–polyelectrolite interaction is complicated by the fact that the dye itself shows a metachromatic behaviour due to its tendency to create aggregates. This is the reason why we first investigated such a phenomenon and, on the basis of the results obtained, we tried to interpret the TB/Hep interaction. Our experiment consisted of a spectrophotometric titration of polysaccharide acidic (pH = 2.06) water solutions by means of TB. As an example in Fig. 4 results obtained for solutions at different Hep concentrations are reported: the Hep concentration refers to the polyanion repeating unit depicted in Fig. 1, having a molecular weight of 974 Dalton. Instead of representing the time on the abscissa we have reported the molar ratio R = TB/Hep between the number of dye molecules added to the solution and the number of the Hep polymeric chains' repeating units.

As may be seen in Fig. 4a, as an example, the three curves depict the absorbance variations at the three wavelengths characteristic of the monomer (610 nm), dimer (580 nm) and trimer (550 nm) of TB [21] as a function of *R*. While the two curves of monomer and dimer just show a point of inflection at nearly an *R* value of 5, the curve of the trimer shows it much later, around $R = 12$ as evidenced by the inspection of the derivative curves. During the experiment the solution remains clear up to this *R* value. At this point a small quantity of a dark blue precipitate was formed which, however, did not seem to increase in amount with increasing the number the TB molecules in the solution, as though a partial redissolution phenomenon was occurring. This may explain the absorbance increase for all the three wavelengths also after the above-mentioned *R* value. At higher Hep concentrations (Fig. 3b–d), the first inflection point becomes progressively more difficult to detect, while that shown by the trimer curve, corresponding to the formation of a precipitate, becomes a gradually more pronounced minimum. The first inflection on the monomer and dimer curves could be attributed to the binding of individual and aggregated TB molecules to the polysaccharidic chain in solution (a titration phenomenon), while the minimum on the trimer curve could be assigned to the aggregation (a precipitation phenomenon) of different Hep/TB chains on which the dye molecules act as a cross-linker.

Fig. 5 illustrates the *R* variation for both the "titration" and the "precipitation" phenomena as a function of the Hep concentration.

As may be seen, while for the titration curve the experimental points may be found just for the Hep dilute solutions up to $C_{Hep} \cong 3 \times 10^{-6}$ M, for the precipitation two different "regimes" may be recognized corresponding to the two Hep concentration ranges below and above $C_{Hep} \cong 5 \times 10^{-6}$ M. All the experimental data in Fig. 5 have been interpolated with power functions which will be discussed later. It is interesting to note that the low Hep concentration conditions favour the aggregation of several dye molecules on the same chain, reaching the formation of a proposed tetrameric stack of dye molecules on the polysaccharide chain in the case of the $C_{Hep} = 3.8 \times 10^{-7}$ M solution. In this case nearly 20 dye molecules are bound to the 5 titrable functional groups present on the Hep repeating unit, while for the $C_{Hep} = 2.9 \times 10^{-6}$ M the

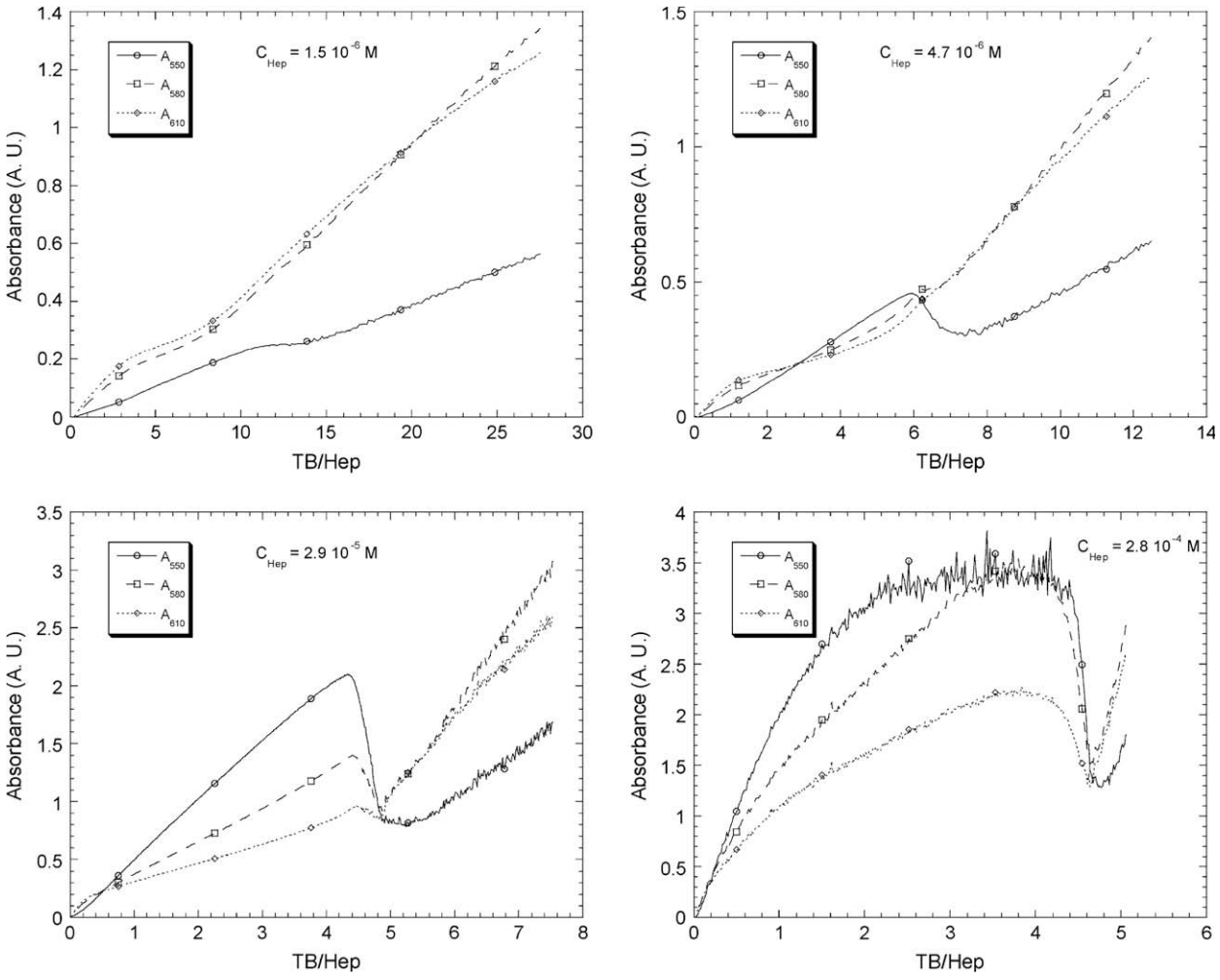


Fig. 4. Absorbance variations of TB at 610 nm, 580 nm and 550 nm as a function of the molar ratio *R* = TB/Hep.

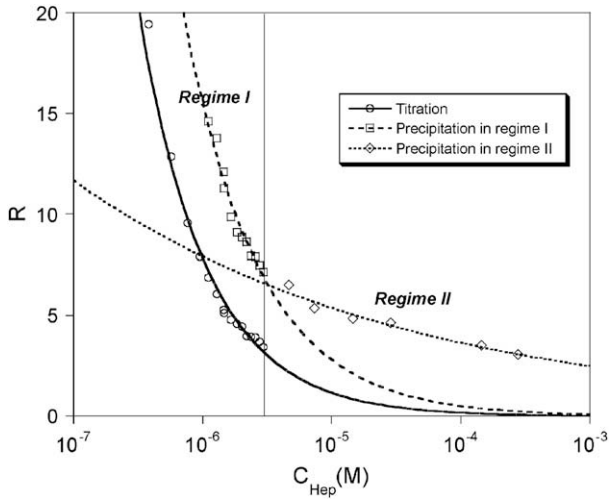


Fig. 5. *R* variation for both the "titration" and the "precipitation" phenomena as a function of the Heparin concentration.

number of bound dye molecules is nearly 3. The low Hep concentration range appears to be unfavourable to the metachromatic reaction. The titration is in fact reached at an *R* value which is at least three times the number of the charged groups present on the Hep repeating unit. Probably the dye molecules prefer to aggregate to each other in solution rather than to interact with the polyanion. A reason for this could be the statistically lower number of useful dye/Hep collisions compared to the dye/dye ones. On the contrary, in the high Hep concentration range the dye molecule sees the polyanion chain already covered by the bound TB molecules. The most probable interaction will then be with the bound molecules rather than with the isolated ones in solution.

The precipitation too, as may be seen in Fig. 5, is strongly dependent on the polysaccharide concentration. It is worth noticing how such a phenomenon is governed by two different mechanisms across the $C_{Hep} \cong 5 \times 10^{-6}$ M. In fact, one can interpolate the precipitation experimental data, below and above this Heparin concentration value, with two different power functions. Such a difference in the precipitation mechanism may be related to different structural forms of the solid Hep/dye complex, a feature that will be further investigated in our laboratory. As may be seen in Fig. 5 the power function interpolating the titration data may be written as

$$\left(\frac{C_{TB}}{C_{Hep}}\right)_{tit} = 8.3 \times 10^{-5} \times C_{Hep}^{-0.8} \quad (1)$$

By solving respect to C_{TB}, the following equation is obtained

$$(C_{TB})_{tit} \cong k \times C_{Hep}^{0.2} = k \times C_{Hep}^{1/5} \quad (2)$$

which evidences how the TB and Hep concentrations are related by the 1/5 power. Indeed in the pH conditions of our experiment (pH = 2.06) the number of functional sites available to the dye on the polysaccharide repeating unit is five.

As far as the precipitation point is concerned, a similar analysis can be done. In the case of Regime I the power function was

$$\left(\frac{C_{TB}}{C_{Hep}}\right)_{prec\ I} = 5.3 \times 10^{-4} \times C_{Hep}^{-0.8} \quad (3)$$

which is fairly similar to the titration equation, giving again a 1/5 ratio of the Heparin repeating unit number to the number of TB bound molecules.

In the case of Regime II

$$\left(\frac{C_{TB}}{C_{Hep}}\right)_{prec\ II} = 7.7 \times 10^{-1} \times C_{Hep}^{-0.2} \quad (4)$$

which results in the following equation by solving with respect to C_{TB}.

$$(C_{TB})_{prec\ II} \cong k \times C_{Hep}^{0.8} = k \times C_{Hep}^{16/20} \quad (5)$$

It is quite surprising that the precipitation in Regime I and the titration phenomenon are described by a similar equation. This can be interpreted by assuming that the precipitation would occur once all the available sites of the polyanion have been saturated (see Fig. 6).

On the other hand in the case of the Regime II precipitation, the Hep concentration being high, the addition of nearly one dye molecule per chain (see Eq. (5)) would be enough to promote the aggregation among two chains, probably by using simultaneously both the amino- and the dimethylamino-groups on the opposite sides of TB.

Since the dye/polyanion interaction is mainly governed by eloctrostatic forces, the influence of the pH of the solution on the above-mentioned phenomena was also investigated. Fig. 7 shows the spectra of TB/Hep solutions ($C_{Hep} = 8.585 \times 10^{-5}$ M) in acidic, neutral and basic conditions for three different values of the ratio *R*, while Fig. 8 shows the variation with pH of the absorbance measured at 610 nm (monomeric TB aggregation state), 580 nm (dimeric TB aggregation state) and 550 nm (trimeric TB aggregation state) for the same *R* values.

As may be seen the effect of the pH is to increase the absorbance of the monomer increasing the solution basicity while that of the dimeric and trimeric aggregates decreases when compared to that of the monomeric species. It is well known that the basic conditions favour the dye aggregation, at least for concentrations of TB higher than 10^{-6} M, although the alkaline pH makes the solutions unstable due to the higher dye reactivity. Moreover, as far as the polysaccharide is concerned, the alkaline pH facilitates the dissociation of the carboxylic groups present on the polymeric chain, making them available to the binding of the TB molecules. In the case of the

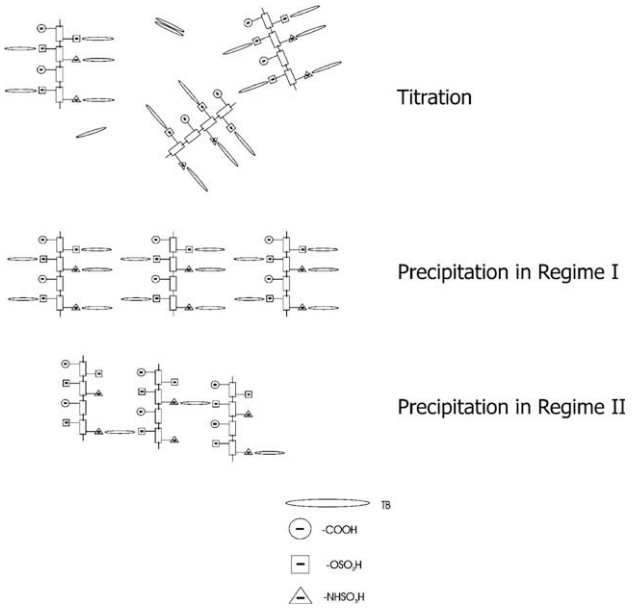


Fig. 6. Molecular model of the TB/Hep interaction in the titration conditions and in the Regime I and Regime II precipitation conditions.

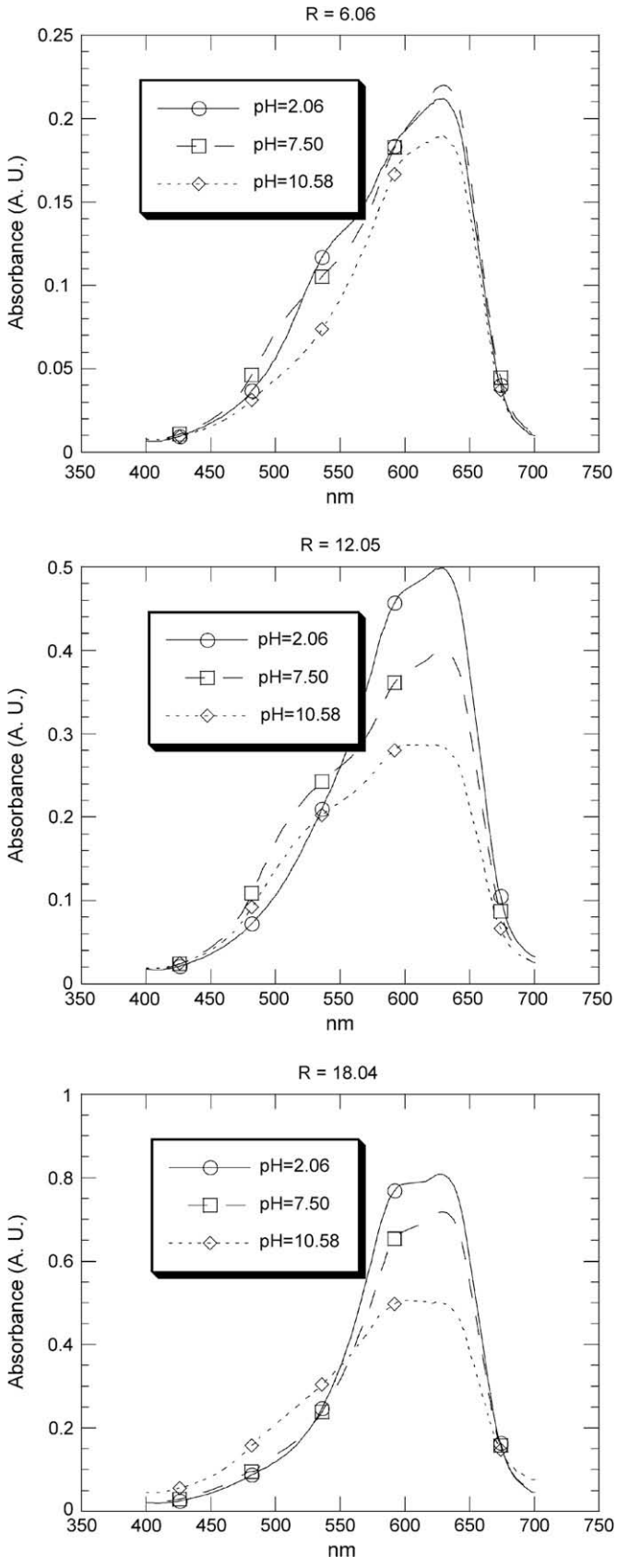


Fig. 7. Spectra of TB/Hep solutions ($C_{Hep} = 8.585 \times 10^{-5}$ M) in acidic, neutral and basic conditions for three different values of the ratio *R*.

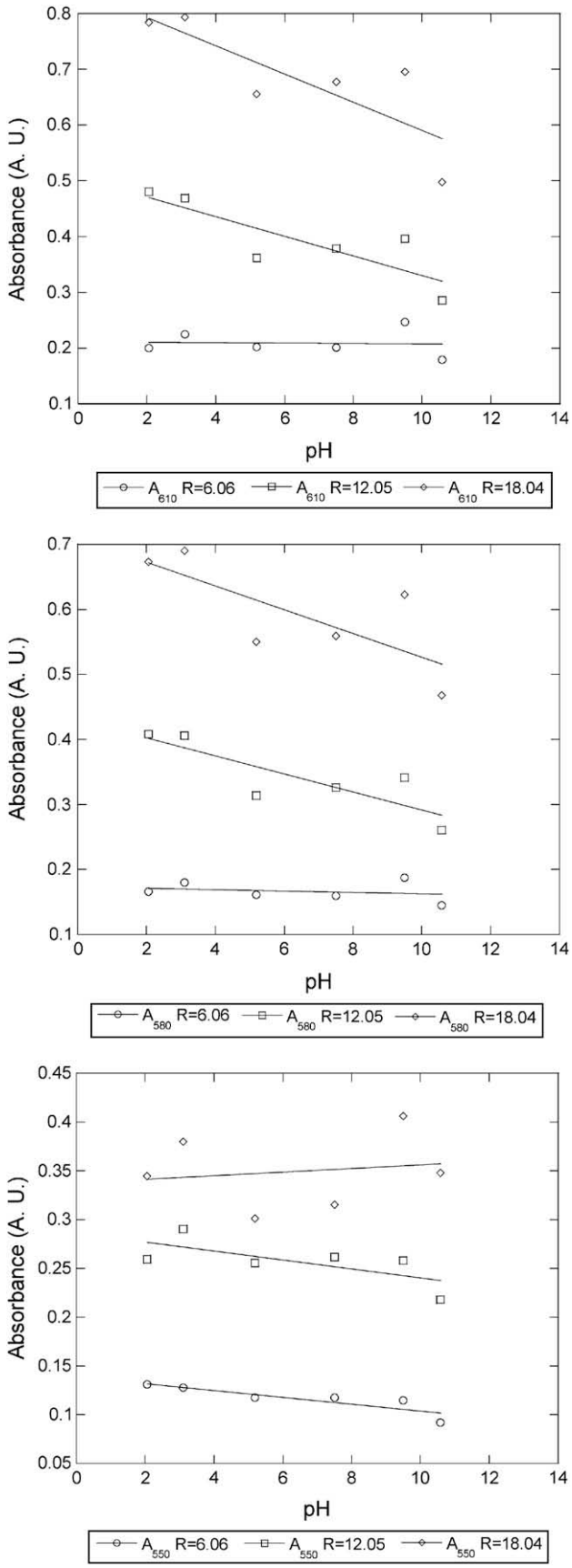


Fig. 8. Variation with pH of the absorbances measured at 610 nm, 580 nm and 550 nm for the same *R* values.

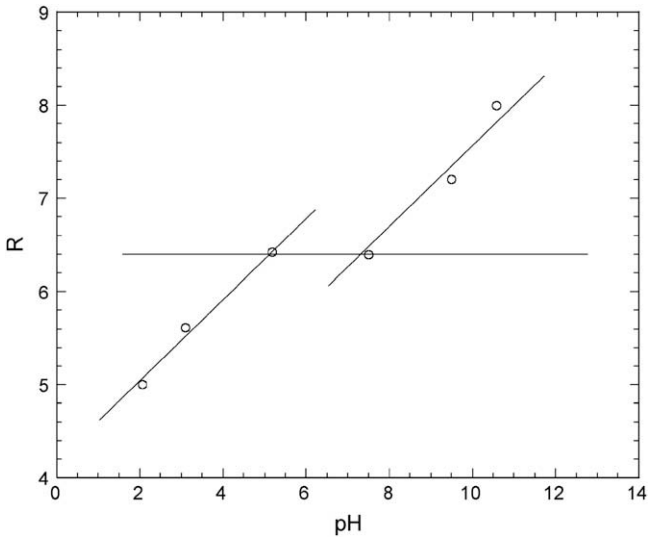


Fig. 9. R variation for the titration phenomenon as a function of pH for the $C_{Hep} = 8.585 \times 10^{-5}$ M solution.

TB/Hep solutions, however, increasing the pH the metachromatic effect is decreased, due to the higher dye "dilution" along the polyanionic chain, which is especially evident for the low R values. On the other hand in precipitation conditions the TB/Hep solution absorbance may increase because of the already mentioned effect of the dye resolubilization by the dye molecules free in solutions. In fact, in basic conditions, when the aminic groups present on the dye molecule are not protonated, the interaction between the dye molecules, is stronger, so favouring the attainment of the highest aggregation levels.

Fig. 9 shows the R variation with pH of the $C_{Hep} = 8.585 \times 10^{-5}$ M solution for the titration phenomenon.

As may be seen, the titration R increases with pH. It is worth noting that there are 7 acidic functional groups present on the Hep repeating unit and that in the basic environment also the two carboxylic groups are dissociated. Therefore the above-mentioned argument, regarding the coexistence on the Hep macromolecule of functional groups characterized by different dissociation constant, may explain the phenomenon which appears to be a discontinuous variation evidenced by a pronounced point of inflection centered at around pH = 6. Moreover the variation with the pH of the dye aggregation properties in solution may contribute to the interpretation of the recorded effects.

4. Conclusions

Although many aspects of the polyanion/dye interactions which influence the chromatic change have already been intensively studied, to date many other aspects remain not discussed. In fact, when adopting a Heparin determination method as that of Smith [12], besides the pH effect, the relative amount of dye and polysaccharide as well as the precipitation of the Hep/dye complex should be taken in great consideration. As we have shown, the latter phenomenon could not be detected in some cases due to the partial resolubilization of the dye, greatly influencing its analytical determination. Moreover the application of our kinetic determination of the polyanion titration point avoids the use of calibration curves which often introduces uncertainty.

Besides the analytical aspects of the present work the physicochemical relevance of the precipitation of the dye/polysaccharide aggregate has also attracted our attention leading us to propose a double regime for this phenomenon connected to different aggregation structures depending on the relative dye/Hep concentrations.

Acknowledgements

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