

# Estimating molecular mass of the complex formed by the B transferrin binding protein (TbpB, Strain CU385) from *Neisseria meningitidis* and human transferrin using gel filtration chromatography and dynamic light scattering

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## ABSTRACT

Transferrin is an 80 kDa serum glycoprotein functioning as a ferric ion carrier in higher eukaryotes. The importance of iron for the life of most organisms has led to the development of mechanisms by which they isolate, transport and store this metal in a safe and efficient manner. Some bacteria like *Neisseria meningitidis* acquire iron by a receptor that binds directly to the transferrin molecule. This receptor consists of two proteins expressed at the bacterial cell surface, TbpA and TbpB. Here, the TbpB-human transferrin complex was prepared and its molecular mass (230 kDa) was estimated using a calibrated gel filtration column. The behavior of this complex was also studied using dynamic light scattering. It was concluded that one TbpB molecule and two transferrin molecules form this complex at physiologic pH. This conclusion is based on the amount of protein used to form the complex. The molecular mass of the complex estimated by gel filtration chromatography was the same as that estimated using dynamic light scattering.

**Keywords:** transferrin receptors, *Neisseria meningitidis*, transferrin binding protein, gel filtration chromatography, dynamic light scattering

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## RESUMEN

**Estimación de la masa molecular del complejo formado por la proteína unidora de transferrina B, cepa CU385 y la transferrina humana utilizando la cromatografía de gel filtración y la dispersión dinámica de la luz.** La transferrina es una glicoproteína del suero que funciona como portadora del ión férrico en los eucariotas superiores. La importancia del hierro para la vida de los organismos vivos ha hecho que éstos desarrollen diferentes mecanismos para aislarlo, transportarlo y almacenarlo de manera eficiente y segura. Algunas bacterias como *Neisseria meningitidis* adquieren el hierro mediante un receptor que se une directamente a la transferrina. Este receptor está constituido por dos polipéptidos TbpA y TbpB que se expresan en la superficie de la bacteria. En este estudio el complejo de la proteína unidora de transferrina B con la transferrina humana es formado y su masa molecular fue estimada (230 kDa) mediante una columna calibrada de gel filtración. El comportamiento de este complejo es también estudiado utilizando la dispersión dinámica de la luz. Se concluye que este complejo está formado por dos moléculas de transferrina y una molécula del receptor TbpB a pH fisiológico. Esta conclusión se hace en base a la cantidad de proteína utilizada en la formación del complejo. La masa molecular estimada por gel filtración es igual, dentro del error, que la masa molecular estimada mediante la dispersión dinámica de la luz.

**Palabras claves:** receptores de transferrina, *Neisseria meningitidis*, proteínas unidoras de transferrina, cromatografía de gel filtración, dispersión dinámica de la luz

## Introduction

The bacterial receptor for human transferrin is composed of two polypeptides: the transferrin binding proteins TbpA and TbpB (Tbps) [1-5]. The fact that Tbps play a critical role in meningococcal survival during infection and that they are surface-exposed makes them attractive vaccine candidates [2, 6, 7].

TbpB is a surface exposed lipoprotein that is anchored to the membrane by its lipid [8].

Little is known about the interaction between the bacterial receptor and the human transferrin. The crystal structure of the ectodomain of the human transferrin receptor has been recently determined [9] and it is known that the human transferrin receptor assists the iron uptake into vertebrate cells through a

cycle of endo- and exocytosis of the transferrin [10]. In the bacterial counterpart it is well known that TbpA can mediate iron uptake in an energy-dependent process without the internalization of transferrin [11]. However, the contribution of TbpB to the iron uptake process is not yet known. The sequence alignment obtained for the known TbpB proteins, from different bacteria isolates, suggests that no homologous protein with known three-dimensional structure has been reported so far.

The availability of several milligrams of pure protein allows this problem to be addressed by crystallizing the proteins alone or forming a complex with human transferrin.

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Here we present the formation, isolation and partial characterization of the complex of the purified TbpB with human transferrin.

The molecular mass of the complex is estimated with a gel filtration calibrated column. It was of 230 kDa and it is consistent with the existence of a trimer formed by one molecule of TbpB and two molecules of human transferrin. The proposed stoichiometry is based on the quantification of the protein obtained for the complex and from the quantity of TbpB used to prepare this complex.

The dynamic light scattering studies of this complex are also presented. Using this technique, a mean molecular mass of 249 $\pm$ 27 kDa was obtained, confirming the result of gel filtration chromatography.

## Materials and methods

### TbpB

TbpB was produced in an *E. coli* strain transformed with a plasmid that codifies for the amino acid sequence of natural TbpB without any lipid, fused to 21 amino acids belonging to the N-terminal domain of P64K protein [21]. This protein was extracted, refolded and purified using a transferrin affinity column.

To form the TbpB-transferrin complex commercial human holotransferrin from Sigma (T7748) was used.

### Gel filtration chromatography

A semi preparative gel filtration chromatography was employed to purify the TbpB-transferrin complex. An empty column was packed in the lab with 200 mL of Sephacryl 200 gel from Pharmacia and it was equilibrated in 20 mM Tris-HCl buffer pH 7.2 and NaCl 0.12 M. The flow rate was 0.2 mL/min and fractions of 4 mL were collected. A prepacked Superdex 200 HR 10/30 column was used to estimate the mass of the complex. The column was equilibrated in 20 mM Tris-HCl buffer pH 7.2 containing 0.15 M NaCl. A flow

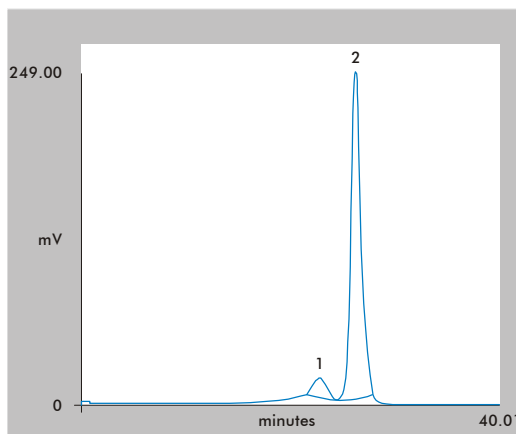


Figure 2. Gel filtration chromatogram of the commercial human transferrin. Peak 2 corresponds to the transferrin monomers that were deglycosylated with PGNase F to from the complex with TbpB. Peak 1 corresponds to transferrin oligomers. The y axis is presented in mV because we converted the ultraviolet analog detection into digital results by means of a computer. The same consideration was made in Figures 4 and 5.

rate of 0.5 mL/min was used. The following molecular markers were used to calibrate the column: Ferritin, 440 kDa; Catalase, 240 kDa; Aldolase, 158 kDa; Human Transferrin, 81 kDa; Bovine Serum Albumin, 68 kDa; Ovalbumin, 43 kDa; Chymotrypsinogen, 25 kDa and Ribonuclease A, 13.7 kDa.

The chromatographic detection was done with a LKB 2158 ultraviolet detector at 280 nm.

### TbpBrec-Human Transferrin Complex formation and purification

The transferrin used contains 2% of aggregates of the same transferrin that was separated from transferrin monomers using the Superdex 200 HR 10/30 column

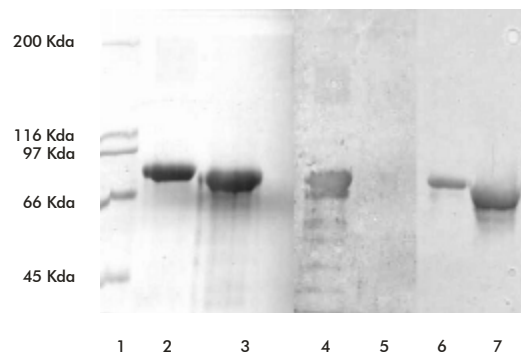


Figure 1. SDS-PAGE and Western Blotting results of commercial and deglycosylated human transferrin. Lanes 2 and 3 are the commercial and deglycosylated human transferrin, respectively. Lane 1 corresponds to the molecular weight markers. Lanes 6 and 7 are the results of Western Blots of the commercial and deglycosylated human transferrin, respectively using a monoclonal antibody against human transferrin. Both bands were recognized by the antibody. Lanes 4 and 5 are the results of a Western Blot made on the same samples, i.e. commercial (lane 4) and deglycosylated human transferrin (lane 5), but using a Concanavalin A-peroxidase as the binder. Signal is only seen for the commercial transferrin sample. This means that the deglycosylated human transferrin does not have any sugar left.

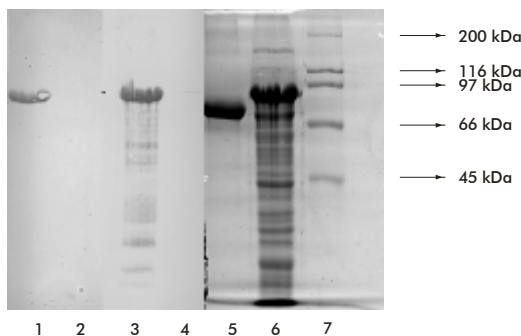


Figure 3. SDS-PAGE and Western Blotting results of the TbpB used in the present work. Lane 6 has the proteins of a crude extract from *E. coli* cells used to produce TbpB. The expression in this case was high and the band of TbpB is at the height of a 90 kDa protein, approximately. Lane 5 is the commercial human transferrin used as a negative control. Lane 3 is the result of a Western Blot of TbpB using a monoclonal antibody against this protein. Lane 4 is the Western Blot result of commercial human transferrin using the same antibody and no signal is observed. Lane 1 is the result of the Western-blot experience of TbpB using transferrin peroxidase. As TbpB is a receptor of human transferrin, a signal was observed. Lane 2 corresponds to the Western-blot, of commercial human transferrin using transferrin-peroxidase as the binder and no signal is observed.

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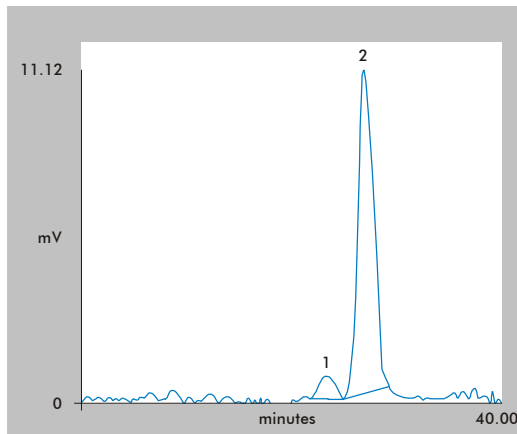


Figure 4. Gel filtration chromatogram of TbpB from *Neisseria meningitidis*, strain CU 385. Peak 2 corresponds to the monomers used to form the complex with human transferrin in the present study. Peak 2 corresponds to TbpB dimmers.

before forming the complex. The transferrin employed was deglycosylated with PGNase F from New England Biolab for a further use of the complex in crystallization trials. The complex formation was analyzed by SDS-PAGE, Dot and Western Blotting techniques.

#### SDS-PAGE analysis and electro blotting

Samples from the purification steps were analyzed by SDS-PAGE using 7 or 10% acryl amide gels and the discontinuous buffer system of Laemmli [22]. Gels were stained with Coomassie R-250 brilliant blue. The monoclonal antibodies against TbpB and human transferrin were kindly provided by the Meningitis Group at CIGB. The transferrin-peroxidase was purchased from Jackson ImmunoResearch, USA.

#### Protein determination

The protein determinations were made with a BCA Protein Assay Reagent Kit from Pierce. The calcu-

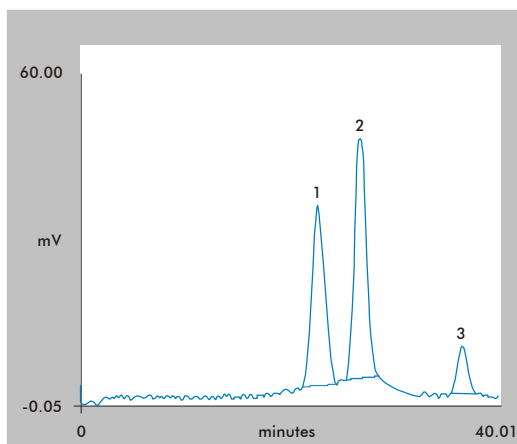


Figure 5. Analytical gel filtration chromatogram of the separation of the TbpB-human transferrin complex from the excess of transferrin. Peak 1 elutes at 22 min and corresponds to the TbpB-human transferrin complex. Peak 2 elutes at 26 min and corresponds to human transferrin. The molecular weight of the complex determined using the same column after calibration was 230 kDa.

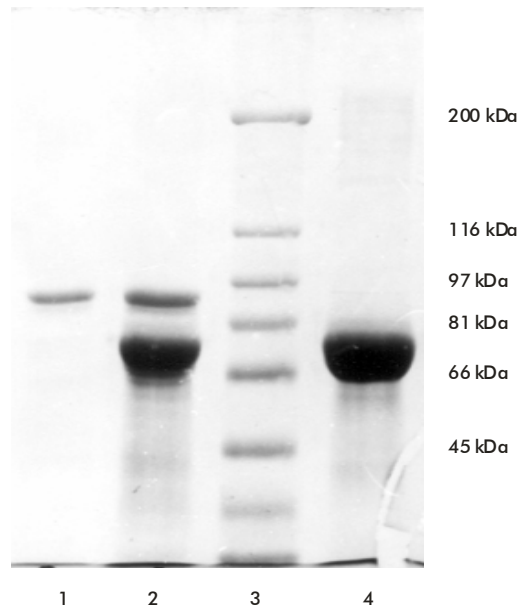


Figure 6. SDS-PAGE results corresponding to the TbpB-human transferrin complex gel filtration separation. The chromatogram of this separation is shown in Figure 5. Lane 4 is the fraction collected under peak 2 and corresponds to the excess of transferrin. Lane 1 corresponds to TbpB. Lane 2 is the fraction collected under peak 1 and corresponds to the TbpB-human transferrin complex formed. The molecular weight markers are presented in lane 3.

lated quadratic linear correlation coefficient for the concentration of BSA protein vs. Absorbance (562 nm) was 0.9978.

#### Light Scattering

To perform the light scattering studies a commercial equipment DynaPro-MS/X [23] was used. The complex formed, as described before, was diluted up to 0.1 mg/mL in 20 mM Tris-HCl buffer pH 7.2.

## Results and discussion

### TbpB Transferrin Complex formation

A commercial human holotransferrin was employed and it was deglycosylated with PGNase F to use this complex for further crystallization trials. Figure 1 shows the results of 7% SDS-PAGE and Western Blot using the commercial and deglycosylated human transferrin.

Figure 2 shows the human transferrin gel filtration chromatogram using the high resolution Superdex 200 10/30 column. Elution time was 26 min, corresponding to a mass of 80 kDa. The same result is obtained with the deglycosylated transferrin. It is observed that the mass difference of both transferrins was only evidenced by SDS-PAGE.

Figure 3 shows the results of 10% SDS-PAGE and Western Blot with the TbpB used here. TbpB is at the level of a 90 kDa protein. The theoretical molecular mass for this TbpB was estimated to be 77 kDa. Figure 4 shows the gel filtration chromatogram of TbpB from the affinity column made with a high resolution Superdex 200 10/30 column. Peak 2 eluted at 26 min corresponded to TbpB monomers with a molecular mass of 81 kDa, Figure 3, lane 1. Peak 1 eluted at 23 min,

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corresponded to TbpB dimers. The estimated molecular mass for this TbpB both by theoretical calculation from its amino acid sequence and by gel filtration, leads to the conclusion that TbpB behaves abnormally in the SDS-PAGE analysis.

One mg of the TbpB (previously separated with a transferrin affinity column and gel filtration to eliminate the presence of oligomers) was used to form the complex and it was mixed with 3 mg of the deglycosylated transferrin, previously purified by gel filtration chromatography to eliminate aggregates. The mixture was incubated for 4 hr at 4 °C.

The complex was separated from the excess of transferrin using a gel filtration semi preparative Sephacryl 200 column from Pharmacia and 2 mg of the complex was obtained. This corresponded to 75% of the expected amount of protein from the molecular reaction. Figure 5 is the chromatogram of the same complex reaction but with an analytical HR Superdex 200 10/30 column. Peak 1 is the TbpB-transferrin complex and peak 2 is the excess transferrin. This was deduced from the SDS-PAGE gel from the results of this separation and is shown in Figure 6. Lane 4 was from the fraction collected under peak 2, Figure 5, and it corresponded to transferrin. It appeared at lower values than those expected for non deglycosylated transferrin (near 70 kDa) because of the lost of the carbohydrate chains due to the deglycosylation process. Peaks 1 and 2 in the present chromatogram are sharp enough to consider them as formed by single proteins. The SDS-PAGE result corresponding to peak 2 (Fig. 6), (lane 4) is transferrin alone, without any TbpB. Lane 2 is the fraction collected under peak 1 and it corresponded to the TbpB-transferrin complex.

### Molecular mass estimation by gel filtration chromatography

The calibrated column HR Superdex 200 10/30 was used to estimate the molecular mass of the TbpB and the human transferrin complex. The calibration of the

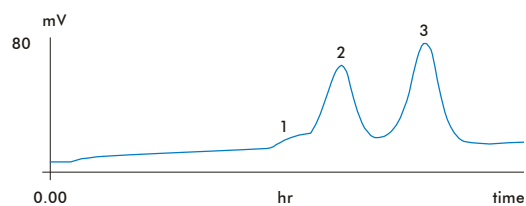


Figure 7. Chromatographic profile of the gel filtration semi preparative separation of the TbpB-transferrin complex made in a 200 ml Sephacryl 200 column packed in the lab. Peak 1 corresponds to aggregates, peak 2 is the TbpB-transferrin complex and peak 3 is transferrin.

Table 1. Final amount of protein for the purified TbpB transferrin complex considering different stoichiometries. Column 2 is the calculated amount of the complex considering 1 mg of TbpB. Column 3 is the same quantity considering a 75% column recovery.

TbpB: Tf	Calculated (mg)	75% Column rec. (mg)
1:1	1.9	1.5+/-0.2
2:1	1.48	1.2+/-0.2
1:2	2.9	2.2+/-0.2

gel filtration column gave a quadratic linear correlation coefficient of the log of the molecular weight with the elution volume of 0.9697. Peak 1 in Figure 5 eluted at 22 min corresponded to the TbpB-human transferrin complex. The molecular mass determined for this complex was 230 kDa, and this value is consistent with the existence of a trimer. Peak 2 in Figure 5, corresponded to human transferrin, eluted at 26 min and the calculated molecular mass using the present calibration was 80 kDa.

### Stoichiometry of the complex

For the stoichiometry of the complex we used the calculated theoretical molecular mass of the present TbpB from its amino acid sequence, which was of 77 kDa. For human transferrin we used the reported theoretical molecular mass of 79.5 kDa [12] and considering the glycosilation to be 6% of the total mass [12], we used 75 kDa as the deglycosilated human transferrin mass.

As mentioned 1+/-0.1 mg of TbpB was used to form the complex, and 2+/-0.2 mg of the complex was obtained from the gel filtration semi-preparative column considering the error of the protein determination to be 10%. Figure 7 is the chromatographic profile of the semi-preparative gel filtration separation of the complex. Peak 1 corresponds to certain aggregation, peak 2 is the complex and peak 3 is transferrin. These results were obtained from an SDS-PAGE analysis of the fractions collected under the peaks of the chromatogram (Fig. 7) with similar results as obtained before with the analytical HR Superdex 200 10/30 column (Fig. 5 and Fig. 6), (gel not shown). Seventy-five percent of the recovery from this column was basically the aggregate formation. The results of the calculation are presented on table I. Only the trimer constituted by two molecules of transferrin and one molecule of TbpB is consistent with the present analysis. The relationship 1:1 is only possible if there was no protein loss in the gel filtration purification, but this consideration was not possible in our experimental conditions as observed in the chromatographic profile of the semi-preparative separation of the complex (Fig. 7).

Table II. Cumulative data log of the results of one of the dynamic light scattering measurements made on the TbpB-human transferrin complex.

Msr #	Time (s)	Amp	Diff Coef	Rad (nm)	Polyd (nm)	Polyd Index	MW (kDa)	%Mass	Temp (C)
1	13	0.851	329	5.92	1.4	0.06	216.3	100	17.2
2	26	0.846	330	5.99	1.5	0.06	222.1	100	17.7
3	39	0.847	325	6.06	1.6	0.07	229.0	100	17.6
4	51	0.839	274	7.19	3.2	0.20	346.1	100	17
5	64	0.854	311	6.34	1.6	0.06	254.9	100	17.6
6	78	0.861	311	6.32	0.8	0.02	253.3	100	17.6
7	90	0.844	312	6.32	1.6	0.06	253.1	100	17.6
8	103	0.832	286	6.88	2.8	0.17	311.7	100	17.6
9	116	0.848	332	5.92	1.2	0.04	216.3	100	17.6
10	128	0.855	308	6.40	2.2	0.11	261.1	100	17.7
11	141	0.852	332	5.90	1.2	0.04	214.4	100	17.4
12	178	0.853	327	5.99	1.6	0.08	222.3	100	17.4
13	190	0.847	308	6.44	2.1	0.10	264.7	100	17.9
14	203	0.849	328	6.05	1.6	0.07	227.8	100	17.9
15	217	0.848	316	6.25	1.3	0.05	246.1	100	17.7
16	228	0.832	294	6.76	1.4	0.04	297.7	100	17.9
Mean Values									
		0.847	314	6.29	1.7	0.08	252.3	100	17.6

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### Dynamic light scattering results

Dynamic light scattering in photon correlation spectroscopy allows us to determine the diffusion coefficient of the particles scattering the light (molecules in this case), from the correlation function generated by the fluctuating intensity of the light scattered.

The theory, techniques and applications of photon correlation spectroscopy have been extensively reviewed [13-16]; however, they will be briefly discussed here for their better understanding.

Fluctuations in intensity are a result of Brownian motions of particles and this motion depends on particle size and shape as well as the temperature and viscosity of the solution.

It has been shown that the photon autocorrelation function from a system of monodisperse particles is given by a single exponential decay. The diffusion coefficient **D** can be found by fitting the measured function to a single exponential. The hydrodynamic radius of particles **R** can be calculated from the known Stokes-Einstein equation:

$$D = kT / 6\pi\eta R$$

Where **k** is the Boltzmann constant, **T** is the temperature, **h** is the viscosity and **R** is the hydrodynamic radius. The molecular weight is derived from this value.

We made three preparations of the TbpB human transferrin complex for dynamic light scattering studies. One of the results obtained is shown on table II. The complex was monodisperse and the mean molecular weight obtained for each preparation was 252.3±37 kDa, 257.9±20.8 kDa and 237.4±23 kDa, respectively. The Dyna Pro equipment, while counting, is programmed to calculate the hydrodynamic ratio and to give other parameters such as temperature, the polydispersion index, the baseline etc. As seen on table I, these calculations were made 16 times. The error was assumed as the square root of the mean quadratic deviation of these values. The mean molecular weight of the three preparations was 249±27 kDa and this value was equivalent to the molecular mass value obtained previously by gel filtration chromatography.

Finally we want to emphasize that all procedures: complex formation, gel filtration and light scattering were performed at physiologic pH (i.e., pH 7.2-7.4).

### General discussion

The fact that one TbpB and two transferrin molecules form the TbpB-transferrin complex implies that TbpB has two regions for transferrin recognition and therefore a bi-lobular structure as suggested by sequence

analysis [17]. There is a previous report [18], in which discontinuous peptides in the TbpB sequence were identified and shown to bind human transferrin. Human transferrin binding regions were identified both in the N and C terminal halves of the TbpB molecule, suggesting more than one recognition site. This TbpB conformation should be related to the need of the pathogen to efficiently acquire iron from the host. This behavior, in solution (*in vitro*), could reflect what occurs in the membrane, *in vivo*.

The present result in which two molecules of transferrin bind to one TbpB molecule contradicts a theoretical dynamic model proposed recently [19] and the result obtained using surface plasmon resonance [20]. A relationship of one molecule of TbpB to one molecule of human transferrin was proposed by these authors. Considering this relationship the molecular mass for this complex should be of 152 kDa. This value is far different from the results obtained here based on two well known analytic techniques generally accepted for molecular mass estimation, such as gel filtration chromatography and dynamic light scattering. One explanation for this disagreement is that the complex formed by two transferrin molecules and one TbpB molecule described here is some kind of intermediary specimen needed for the bacteria to recognize the transferrin.

It will be of great value to measure the complex molecular mass by using another technique, such as MALDI-mass spectrometry. In this sense electro spray mass spectrometry under non-reducing conditions was tested and the spectra were too noisy to allow for any conclusion (data not shown).

### Conclusions

1) A human transferrin-TbpB complex was obtained and its molecular mass was estimated using a calibrated gel filtration column. The molecular mass determined for this complex was 230±23 kDa, corresponding to a trimer. Considering the amounts of protein used in the complex formation it is concluded that this trimer is composed of one TbpB and two transferrin molecules.

2) The complex formed was studied by dynamic light scattering and it was demonstrated that the complex was monodisperse. The mean molecular mass obtained by this technique was 249±27 kDa, and it is equivalent to that obtained by gel filtration chromatography.

### Acknowledgements

The authors are much indebted to Dr. Lesley Haire and Professor Guy Dodson from the National Institute for Medical Research in London, who have been most helpful to us during the present work.

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