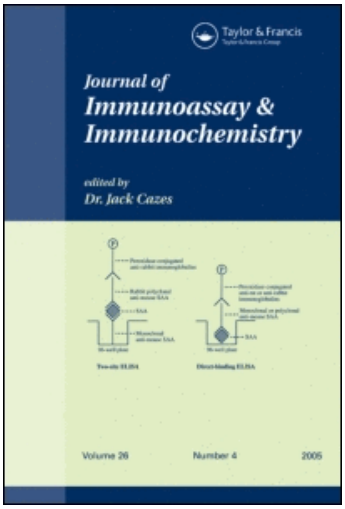

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POLYACRYLIC MICROSPHERES AS A SOLID PHASE FOR MICROPARTICLE ENHANCED NEPHELOMETRIC IMMUNOASSAY (NEPHELIA (R)) OF TRANSFERRIN.

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ABSTRACT

Polyfunctional hydrophilic microspheres (MS) can be produced by copolymerisation with gamma-irradiation of acrylic monomers. Transferrin (TRF) can be covalently bound to these MS by reaction between aldehyde groups of the MS and primary amino groups of the protein. MS-TRF conjugates thus obtained are agglutinated by specific antiserum and this agglutination is inhibited by free TRF. Agglutination and inhibition are quantified by measurement of the light scattered by MS-TRF conjugate clusters with a specially designed nephelometer, a process designated as microparticle enhanced nephelometric immunoassay (NEPHELIA (R)) for TRF. Recovery, correlation and reproducibility studies, simultaneously performed in three different laboratories, show that this TRF immunoassay is accurate for a large concentration range. NEPHELIA (R) may appear as an alternative method for a large variety of molecules.

(KEY WORDS: Microparticle; Microparticle Immunoassay; Immunonephelometry; Transferrin)

INTRODUCTION

Conventional immunonephelometry is an immunoassay based upon the nephelometric quantification of the antigen-antibody reaction: it is an easy-to-perform method, for several years used to measure many proteins in various biological fluids (2,3), but its detection limit (1 mg/L reaction mixture) is poor compared with immunoassays using tracers such as radioimmunology or immunoenzymology.

Several authors have reported the increase in sensitivity of the conventional immunonephelometry by binding antigen or antibody to particles able to amplify the light scattered by the antigen-antibody complexes (4,5,6,7,8,9). However, these reagents, usually made of polystyrene, were often unstable (spontaneous autoagglutination, fast sedimentation) and hydrophobic (non specific protein adsorption and protein releasing). Sonication is required before their use to ensure dispersion of aggregates, and hydrophilic coating or sample pretreatment are necessary to warrant their stability during the assay.

This study reports the synthesis of stable hydrophilic and polyfunctional microparticles and their use as a nephelometric tracer for the antigen-antibody reaction. An improved microparticle enhanced nephelometric immunoassay (NEPHELIA (R) (1)) is described and applied as an example to the measurement of human serum Transferrin (TRF).

MATERIALS AND METHODS

Chemical Reagents

2-Hydroxyethyl methacrylate, acrolein, methacrylic acid, Triton (R) X-100 and polyethylene glycol (PEG) 6000 (MW 5000 to 7000) were purchased from Merck (WG); N,N'-methylene bis acrylamide from Eastman Kodak (US). Sodium dodecyl sulfate, hydroquinone, sodium chloride, sodium dihydrogenophosphate, disodium hydrogenophosphate, 2-aminoethanol, sucrose and sodium azide, of analytical reagent grade, were obtained from Prolabo Rhone Poulenc (FR). The buffer for nephelometry supplied by Diagnostics Pasteur (FR) was used for some assays.

Biochemical Reagents

Purified TRF was a product of the "Centre National de Transfusion Sanguine" (FR); lyophilised TRF (92% pure) was dissolved in distilled water and dialysed against 0.05 M sodium phosphate buffer (PB) pH 7.2 containing 0.33 M NaCl. Anti-TRF goat antiserum (specific antibodies: 6.9 g/L), TRF control serum (3.20 +/- 0.30 g/L) and TRF standard serum (8.7 g/L), were kindly supplied by Diagnostics Pasteur (FR). Human serum samples were randomly chosen from the patients of the Nancy (FR) University

Hospital and of private clinical laboratories; they were stored at +4°C or frozen at -20°C.

Preparation of microspheres

Two suspensions of polyfunctional hydrophilic MS, specially devised for nephelometric immunoassay, were used in this work: they were synthesized by copolymerisation, initiated by gamma irradiation (^{60}Co, 75 krad /cm^2) of aqueous solutions containing 80 g/L and 120 g/L of acrylic monomers (Acrolein 47%, 2-Hydroxyethyl methacrylate 49.7%, Methacrylic acid 2% and N,N'-Methylene bis acrylamide 1.3% of total monomers) with 0.8 g/L and 0.9 g/L respectively of sodium dodecyl sulfate. MS thus obtained were stored several years in Hydroquinone (1 g/L) at +4°C, under argon; their characterisation (shape, dispersion, size, concentration) was carried out as previously reported (10).

Preparation of MS-TRF conjugates

8.87 mg and 0.57 mg of TRF were mixed with 10 mg of MS obtained by copolymerisation of 80 g/L and 120 g/L respectively of monomers, in 1 ml of 0.05 M PB pH 7.2, 0.33 M NaCl. After gentle stirring 18h. at +4°C, 0.05 ml of 2-aminoethanol buffered solution (2.4 M, pH 8) was added and unreacted aldehyde groups of MS were

blocked for 4 h. at room temperature. MS-TRF conjugates were then separated from binding mixture by centrifugation (Spinco L, +4°C, Beckman SW50, 1h., 12000 g and 8000 g) on discontinuous sucrose gradients (200/800 g/L in 0.1 M PB pH 7.2, 0.33 M NaCl). MS-TRF conjugates, collected at the interface of sucrose solutions, were stored at +4°C in 0.1 M PB pH 7.2, 0.33 M NaCl, with NaN_3 2 g/L.

Imunonephelometric study of the MS-TRF conjugates

Intensity of light scattering was measured with the Diagnostics Pasteur (FR) nephelometer (Nephelia N 600) during agglutination of the two MS-TRF conjugates by serial dilution of the anti-TRF antiserum in 0.01 M PB pH 7.2, 0.14 M NaCl with or without 30 g/L of PEG 6000. For nephelometric study of inhibition of MS-TRF conjugate agglutinations, known concentrations (0 to 5 mg/L) of TRF (purified TRF or TRF standard serum) were mixed with MS-TRF conjugates and anti-TRF antiserum at dilutions previously chosen on account of the agglutination results.

Microparticle enhanced nephelometric immunoassay of TRF

Microparticle enhanced nephelometric immunoassay of TRF (NEPHELIA (R)) was simultaneously performed in 3 different

laboratories (Sites A, B and C). NEPHELIA (R) was an one step immunoassay: Standard or control or unknown sera, anti-TRF antiserum and MS-TRF conjugate were mixed together in 1 cm light path measurement microcuvette (Diagnostics Pasteur, FR) according to the procedures described in the Table 1.

Precision was assessed by measuring TRF concentration in largely distributed samples of human sera by 20 (Site C) and 30 (Site B) assays repeated within one day (Within-assay precision) and by assays repeated on each of 15 (Site A) and 10 (Site C) days (Between-assay precision). Recovery study was performed by adding 5 known increasing amounts of purified TRF (1.28 to 7.34 g/L) to different sera (Sites A and B).

For comparison of methods, TRF concentration of human serum samples was measured by conventional immunonephelometry (Behring Laser Nephelometer, WG) at site A of evaluation, by the Array System Beckman (US) at site B and by the RA 1000 System Technicon (US) at site C, according to the manufacturer's recommendations.

RESULTS

MS-TRF conjugates

Two MS suspensions, unlike in their dry diameter, can be produced by copolymerisation of acrylic monomers performed as previously described: 125 nm (SD = 16 nm, n = 43) diameter MS

TABLE 1

Procedures for NEPHELIA R determination of serum TRF used in three sites of evaluation.

	REAGENT	REACTION VOLUME µl	REAGENT DILUTION or CONCENTRATION in REACTION CUVE
S I T E A	6 serial dilutions of Standard Serum or Sample or Control	16	1550 to 48 µg/L 1/6250
	Anti-TRF Antiserum	100	1/1600
	0.01 M PB pH 7.2 0.14 M NaCl Tween 20 0.8 g/L	284	
	MS-TRF conjugate 125 nm diameter	50	200 mg/L
	Nephelometric Measurement after 1h.		
S I T E S B a n d C	MS-TRF conjugate 235 nm diameter	50	42 mg/L
	5 prediluted TRF Standard Sera or Sample or Control	20	3700 to 230 µg/L 1/2350
	Buffer for Nephelometry	350	
	Anti-TRF Antiserum	50	1/16000
	Nephelometric Measurement after 30 min.		

were synthesized from the mixture containing 80 g/L of monomers and 0.8 g/L of sodium dodecyl sulfate with a yield of 63 % ; 235 nm (SD = 6 nm, n = 36) diameter MS were obtained, with a yield of 64 % , when 120 g/L of monomers and 0.9 g/L of surfactant were used. TRF was covalently bound to MS by formation of imine bonds between aldehyde functions of the MS and primary amino groups of TRF (10). In the described binding conditions, uncoupled TRF was not detected in binding supernatant by conventional immunonephelometry and reduction of imine bonds by metallic hydride was not necessary for stability of MS-TRF conjugates.

Immunonephelometric study of the MS-TRF conjugates

MS-TRF conjugates were not spontaneously autoagglutinated and their sedimentation was slow (Decrease of scattered light lower than 6% for 24 h. at rest). Figure 1 shows the nephelometric quantification of MS-TRF conjugates agglutination in the presence of anti-TRF antiserum dilutions: the light scattered by aggregates and the last agglutinating antiserum dilution were greater for the 235 nm diameter MS (450 mV, 1/100 000) than for the 125 nm MS (270 mV, 1/10 000) and their agglutination kinetics was faster. PEG, added to the reaction mixture (30 g/L), allowed an increase of scattered light (270 to 520 mV) during the agglutination of the 125 nm diameter MS-TRF conjugate (Figure 2) but the last

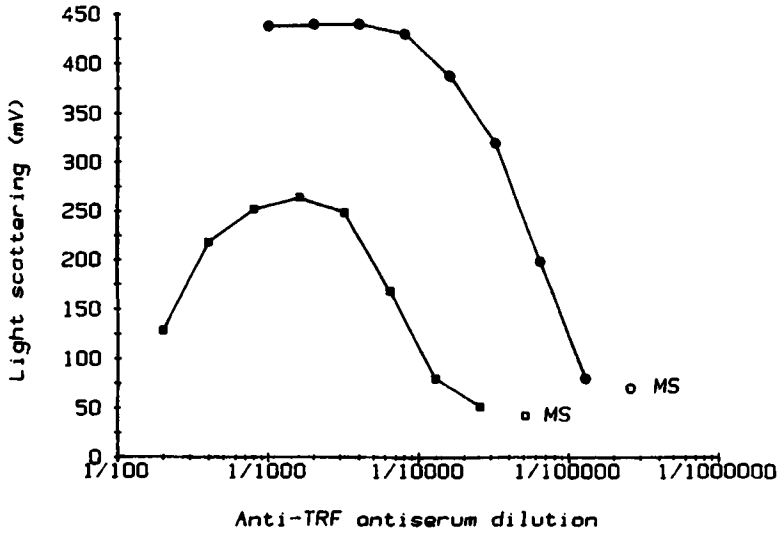

FIGURE 1. Intensity of light scattered, as a function of anti-TRF antiserum dilution, during immunological agglutination of MS-TRF conjugates.
(● 125 nm diameter MS at 200 mg/L; ● 235 nm diameter MS at 42 mg/L; 0.01 M PB pH 7.2, 0.14 M NaCl; reaction time: 1h; MS: intensity of light scattered by the MS-TRF conjugates alone).

agglutinating antiserum dilution was not modified (1/10 000). Inhibition of these agglutinations can be seen in the Figure 3 as a function of free TRF concentration: the 50 % inhibitions were observed for about 300 µg/L of TRF (SD = 9 µg/L, n = 15) with the 125 nm diameter MS-TRF conjugate, with or without PEG added, and for about 800 µg/L of TRF (SD = 18 µg/L, n = 10) with the 235 nm diameter MS-TRF conjugate. In the first inhibitions, 40 µg/L of TRF were signicatively detected in reaction mixture, as giving an intensity of scattered light 3 SD lower than the mean of the intensity of light scattered without TRF.

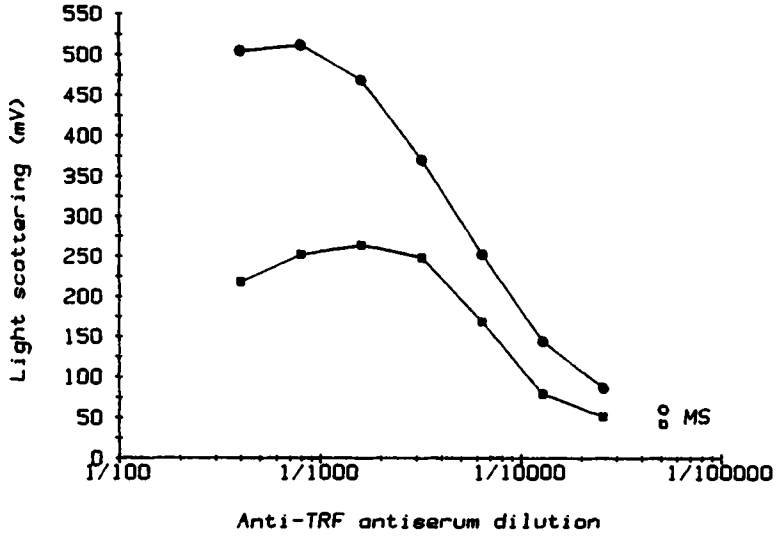

FIGURE 2. Intensity of light scattered, as a function of anti-TRF antiserum dilution, during immunological agglutination of MS-TRF conjugate (125 nm in diameter, 200 mg/L) in presence of PEG 6000 (●) at 30 g/L and without PEG (■).
(0.01 M PB pH 7.2, 0.14 M NaCl; reaction time: 1h; MS: intensity of light scattered by the MS-TRF conjugate alone).

Microparticle enhanced nephelometric immunoassay of TRF

Sigmoidal calibration curves used for TRF assays in the 3 evaluation laboratories are shown figure 4: the assay ranges (0.30 to 9.69 g/L for site A, with the 125 nm diameter MS-TRF conjugate and 0.54 to 8.69 g/l for sites B and C, with the 235 nm MS-TRF conjugate) covered normal (2.0 to 3.2 g/L) and high or low physio-pathological concentrations (11,12,13). Calculated CVs for reproducibility of the calibration curves, on a period of 6

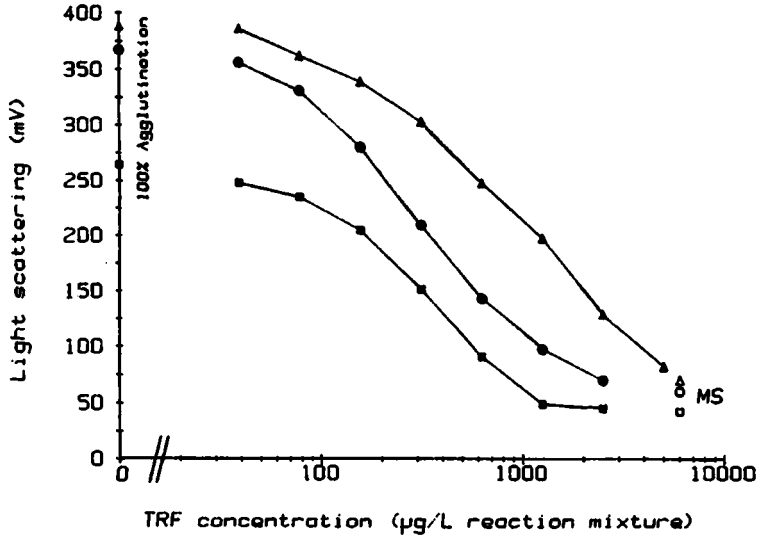

FIGURE 3. Variation of light scattering intensity with TRF concentration in reaction mixture during inhibition by TRF (serial dilutions of TRF standard serum), of MS-TRF conjugate agglutinations.
(■ 125 nm diameter MS-TRF conjugate at 200 mg/L with 1/1600 diluted antiserum; ● 125 nm diameter MS-TRF conjugate at 200 mg/L with 1/3200 diluted antiserum and PEG 6000 (30 g/L); ▲ 235 nm diameter MS-TRF conjugate at 42 mg/L with 1/16000 diluted antiserum; 0.01 M PB pH 7.2, 0.14 M NaCl; reaction time: 1h; MS: intensity of light scattered by the MS-TRF conjugates alone).

months, were ranged from 0.7 to 4.3 %. On account of the good sensitivity of the method, serum samples were used highly diluted (1/6250 and 1/2350) for the nephelometric measurements.

The results of precision studies are reported in Table 2: Within-assay CVs ranged from 2.6 to 8.2 % at sites B and C together, for 10 TRF concentrations between 0.66 and 7.79 g/L, and

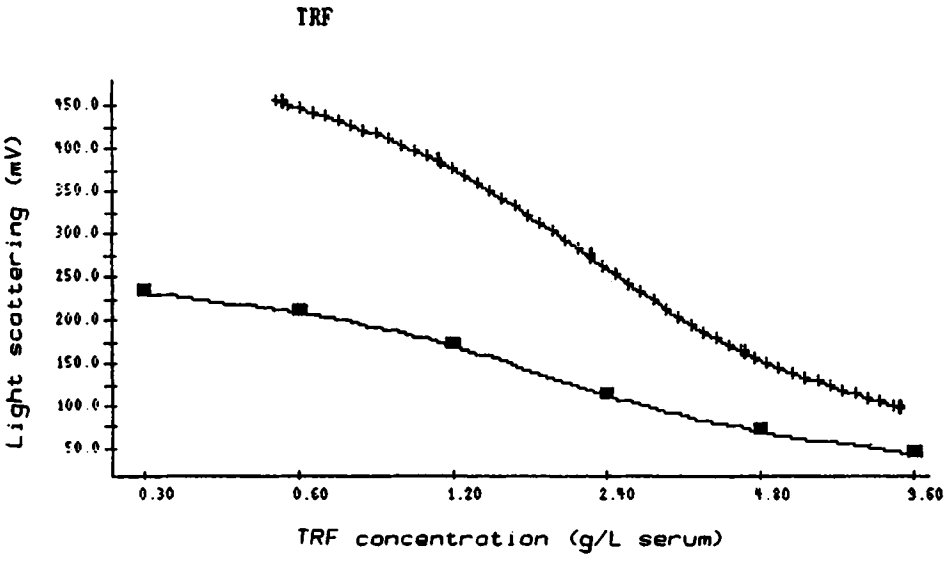

FIGURE 4. Calibration curves for NEPHELIA R of TRF in human serum used at site A (●) and at sites B and C (+). (Assay procedures are given in Table 1)

between-assay CVs were no greater than 6.6 % at sites A and C, in spite of the great dilutions of samples.

Linear recovery profiles were obtained at site B: n = 10, r = 0.99, Recovered TRF = -0.12 + 1.05 Added TRF, mean % = 99.4 %, SD = 9.3 % (Figure 5) and at site A (n = 10, r = 0.99, Recovered TRF = -0.29 + 1.06 Added TRF, mean % = 98.8 %, SD = 2.0 %)

Comparison of NEPHELIA (R) with 3 generally used methods was performed for a total of 138 human sera distributed on a large range of TRF concentration (1.10 to 9.82 g/L). The linear regression parameters calculated for these comparisons are fully reported in the Table 3.

TABLE 2

Within- and Between-assay Precisions of NEPHELIA R for serum TRF: *: 30 assays and • *: 20 assays repeated within a day. Assays repeated on 15 days: • • • and on 10 days: • • • *. ($\bar{C}$ = mean of measured concentrations; SD = standard deviation; CV = coefficient of variation)

	$\bar{C}$ (g/L)	SD (g/L)	CV (%)
SITE B	1.13	0.08	7.1
*	1.79	0.13	7.3
	2.20	0.10	4.5
	3.02	0.22	7.3
	4.23	0.32	7.6
SITE C	0.66	0.04	5.6
* *	1.06	0.09	8.2
	2.01	0.09	4.3
	4.19	0.12	2.8
	7.79	0.20	2.6
SITE A	3.25	0.17	5.2
* * *	8.62	0.45	5.3
SITE C	2.21	0.13	6.0
* • • •	4.51	0.16	3.5
	7.25	0.48	6.6

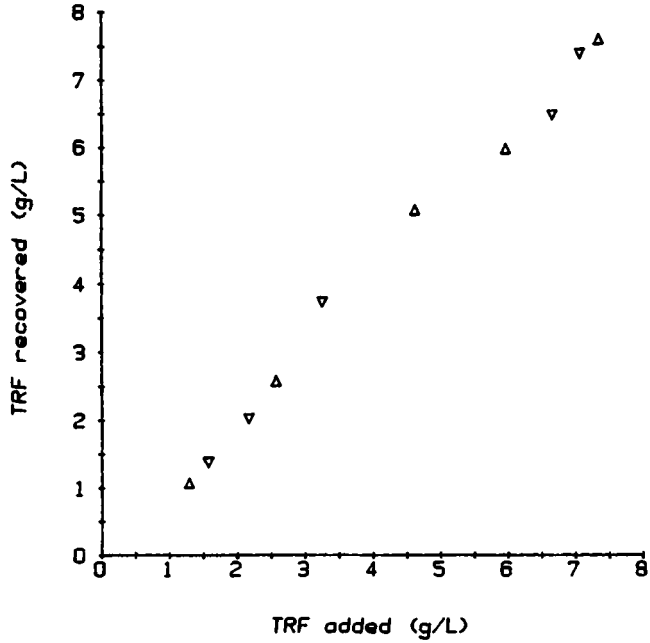

FIGURE 5. Analytical recovery of TRF observed with two overloaded sera (▲ and ▼) at site B of evaluation.

DISCUSSION

Despite their excellent sensitivities, the immunonephelometric assays using particle amplification have not been used extensively (14) owing to the inadequacy, largely due to their hydrophobicity, of the particular reagents previously available. Preliminary studies performed in our laboratory (15,16,17) have shown the great interest of hydrophilic particles for these immunonephelometric assays. The particle synthesis,

TABLE 3

Comparison of Methods by Linear Regression Analysis: NEPHELIA = b + a Compared Method; n = number of tested samples; r = correlation coefficient.

		SITE A	SITE B	SITE C
COMPARED METHOD		Behring Laser Nephelometry	Array System Beckman	RA 1000 Technicon
CONCENTRATION RANGE		1.77 to 9.82 g/L	1.43 to 5.12 g/L	1.10 to 4.74 g/L
LINEAR REGRESSION PARAMETERS	n	44	60	34
	r	0.97	0.86	0.94
	b	0.04 g/L	0.59 g/L	0.21 g/L
	a	0.91	1.18	1.03

described in this paper, produces MS specifically conceived for that use (Manuscript in preparation): they are hydrophilic (Hydroxyl and carboxyl functions), stable (charged carboxyl groups) and covalently coated (aldehyde groups) with ligands containing amino groups. Their diameter can be predetermined by choosing the synthesis conditions such as total concentration of monomers, relative concentrations of each monomer and of surfactant.

MS-TRF conjugates, obtained by binding TRF to these MS, are agglutinated by anti-TRF antiserum: the light scattered by MS-TRF conjugates aggregates can be quantified with a specially designed

nephelometer for antiserum dilutions range where classical immune complexes, formed by the antigen-antibody reaction without MS, are too small to sufficiently scatter the light. The intensity of light scattered by the MS-TRF conjugates agglutination and the agglutinating antiserum dilution increase when the diameter of MS changes from 125 nm to 235 nm: the aggregates of 235 nm diameter MS-TRF conjugate are bigger and have a still sufficient dimension to intensely scatter the light, when obtained for high antiserum dilutions (1/100 000). Therefore, the MS behave as perfect nephelometric tracers of the antigen-antibody reaction by amplifying light scattered by the immune complexes. PEG 6000 has been often used in conventional immunonephelometry for its enhancing effect on the immunological reaction (18,19): in this study, PEG 6000 (30 g/L) allows a better amplification of light scattered during the MS-TRF conjugate agglutination but does not increase its sensitivity. The complete inhibition, by free TRF, of the MS-TRF conjugates agglutinations comfirms the specificity of those and shows that the MS-TRF conjugates can be used as reagents in a sensitive microparticle enhanced nephelometric immunoassay (NEPHELIA (R)) for TRF.

NEPHELIA (R) is particulary easy to perform: it is a one step immunoassay without washing or phase separation. The high dilution of human serum samples avoids interference from turbic samples and no sample blank measurement nor sample pretreatment are required. The assay evaluation, performed in three independent laboratories,

shows that adequate assay ranges for serum TRF quantification were easily covered with accuracy. The TRF assay reported here is only an example of NEPHELIA (R) application: this microparticle enhanced nephelometric immunoassay, with these specifically conceived hydrophilic microparticles, has been also used to perform the determination of many other biological molecules: reports of these applications to immunoglobulins, apolipoproteins, acute phase proteins, hormones are in progress and some NEPHELIA (R) diagnostic kits are already commercialized.

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