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Methodology for assaying recombinant interleukin-2 associated with liposomes by combined gel exclusion chromatography and fluorescence

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Abstract

A simple methodology based on fluorescence and gel exclusion chromatography (GEC) has been developed to assay recombinant Interleukin-2 (rIL-2) associated with vesicles. A Sephadex G75 column was used to separate the liposomes from non-entrapped rIL-2. The elution of the rIL-2 liposomes was monitored by coupling fluorescent and light scattering detection. The solubilisation of the vesicles with octylglucoside (OG) before the assay was necessary to avoid interference from light scattering. This methodology can be automated to yield an on-line system that can separate, solubilise and quantify rIL-2 in liposome samples. It can be extended to any protein associated with vesicles provided that the former can be detected by fluorescence.
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1. Introduction

The cytokine Interleukin-2 (IL-2) has been investigated extensively because of its central role in the regulation of the immune response, including stimulation of T-cell proliferation and antibody production by B cells. The production of recombinant IL-2 (rIL-2) has cleared the way for large-scale clinical applications, notably in oncology and AIDS treatment. However, rIL-2 is a 15-kDa protein which is rapidly eliminated by renal filtration and serious side-effects, including capillary leak syndrome, are associated with the systemic administration of the high doses of rIL-2 necessary to attain effective concentrations. Different systems: minipellets [1], supramolecular biovectors [2], microspheres [3] and liposomes [4–8], have been tested for their ability to increase efficiency and reduce side-effects by increasing the biological half-life of rIL-2.

Liposomes are interesting systems for drug delivery because of their molecular organisation, consisting of one or more phospholipid bilayers enclosing an aqueous cavity in which an active substance can be encapsulated. They can also include hydrophobic molecules with their bilayer membranes, which would be advantageous for rIL-2 association because

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the non-glycosylated protein is very poorly water-soluble. Liposomes have been employed as a delivery system for rIL-2 in systemic [4–6] as well as in locoregional immunotherapy [7,8]. They may function as a depot releasing entrapped rIL-2 over a prolonged period, leading to increased efficiency in vitro and in animal tumour models.

During previous work [9,10], we studied the association of rIL-2 with small liposomes prepared from dipalmitoylphosphatidylcholine (DPPC) by sonication. The amount of associated rIL-2 depended on the lipid concentration and reached levels of loading that could not be solely due to encapsulation of the protein within the internal aqueous phase of the liposomes. Several complementary techniques (calorimetric analysis of lipid interactions, intrinsic fluorescence of tryptophan residues in the protein, protection from enzymatic degradation) showed that rIL-2 was associated with and partially inserted into the lipid bilayer, as would be expected from the hydrophobic nature of the non-glycosylated protein. Similar results were obtained later by Koppenhagen et al. [11] for the adsorption of rIL-2 (from a different source) onto liposomes with different lipid compositions.

In the work referred to above, rIL-2 associated with liposomes was quantified by high-performance liquid chromatography (HPLC) [10,12], after separation of free and associated protein by ultrafiltration [10] or density gradient centrifugation [11]. However, separation by gel exclusion chromatography (GEC) as already described by Fleury et al. [10], would be a more appropriate method for preparing larger quantities of liposomes for biological assays. In this context, monitoring rIL-2 elution from the column by HPLC would be time-consuming and would use up a large proportion of each fraction. We have therefore developed a method of detecting and quantifying rIL-2 based on the intrinsic fluorescence of the single tryptophan residue within the protein molecule. In this article, we present this method and compare its accuracy and precision with those of HPLC. The use of on-line fluorescence and light scattering detection to monitor elution of rIL-2 and liposomes from a GEC column is described. Since the intrinsic fluorescence of the protein is modified by association with lipids, it is necessary to disrupt the liposomes before quantifying the associated rIL-2; this method is also validated. Quantification of rIL-2 by fluorescence is rapid and uses only a small sample; furthermore, in the case of free rIL-2, the sample can be recovered.

2. Experimental

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N*-Octyl β,D glucopyranoside (OG) was obtained from Sigma (St Louis, MO, USA), sodium hydroxide from Prolabo (Paris, France). Recombinant IL-2 (rIL-2) (RU 49637) produced in *Escherichia coli* was provided by Roussel-Uclaf (France).

2.2. Interleukin-2 purification

It should be noted that solubilisation of non-glycosylated IL-2 can only be obtained at low pH (pH≤4.5) or by using detergents like sodium dodecyl sulfate (SDS). SDS needs to be eliminated before liposome preparation and the pH must be adjusted to neutrality to ensure liposome stability.

Recombinant IL-2 was obtained as a freeze-dried powder in capped bottles each containing 10^7 IU (916 μg) rIL-2, 9 mg citric acid and 40 mg Dextran® 70, to which addition of 1 ml of purified water yielded a solution of recombinant IL-2 at pH 3.

The presence of the dextran used as lyophilisation/solubilisation excipient might have perturbed encapsulation of the protein and stability of the liposomes by creation of an osmotic gradient between the external and the internal compartments of the liposomes. It was therefore necessary to remove this additive by selective precipitation of rIL-2 at pH 5.5 after addition of NaOH solution. The precipitated rIL-2 was separated from dextran in solution by centrifugation (Sigma 3K30, Bioblock Scientific, Germany) (10 000 rpm, 10 °C, 10 min), (rotor no. 11134, swing-out head for 4×10 ml with buckets, max. radius 9.6 cm, min. radius 2.6 cm) in Nalgene® Ultralok tubes (Nalge, Rochester, NY, USA). The protein was washed twice with water and vacuum-dried overnight (Leybold, Germany). The recovered rIL-2 was dissolved in citric acid solution (9 g l^{-1}; pH 3).

2.3. Liposome preparation

DPPC (10 mg) was dissolved in chloroform (about 0.5 ml) and deposited as a film on a flat-bottomed flask by slow evaporation under nitrogen followed by overnight vacuum drying. This film was hydrated with 3 ml rIL-2 solution (250 μg ml^{-1}, pH 3). The multilamellar vesicles (MLV) formed were homogenised by bath sonication for 1 min at 50 °C (Branson 2200, Branson Ultrasonics, Danbury, USA). Size reduction of liposomes was carried out by two different procedures: sonication or extrusion, as follows.

Small unilamellar vesicles (SUV) were prepared by probe sonication at 38 °C using a Vibracel 600 W ultrasound generator (Bioblock Scientific) at about 90 W power and 50% cycle time. Five sonication cycles of 2 min were each separated by 1 min of rest. Ultrasound generates local heating and the suspension temperature was in fact higher than the DPPC phase transition temperature of 41 °C. Titanium particles generated by sonication were removed from the liposome suspension by centrifugation at 4500 rpm, for 5 min at 10 °C (rotor no. 12158, angle rotor 6×30 ml, max. radius 7.9 cm, min. radius 2.3 cm, angle 30°) in a Nalgene tube (Nalge, Rochester, NY, USA).

Large unilamellar vesicles (LUV) were prepared at 50 °C by successive extrusions under nitrogen pressure (max. pressure=3000 kPa) of the MLV suspension. The latter was passed twice through each of successively 0.8, 0.4, 0.2, 0.1, 0.05 μm polycarbonate filters (Poretics, Livermore, USA).

The mean particle diameter was estimated by quasi-elastic light scattering (Nanosizer N4MD, Coulter Electronics, FL, USA) using the ''unimodal'' method of date processing. The samples were analysed at 25 °C; the viscosity and refractive index of the aqueous suspending medium were taken to be 0.890 mPa s and 1.3325, respectively. The mean diameters of liposomes obtained by sonication and extrusion were 49 and 104 nm (the standard deviations of one normally distributed dispersion were 9 and 16 nm), respectively.

2.4. Separation by gel exclusion chromatography

Liposomes were separated from free rIL-2 by exclusion chromatography on Sephadex® G75 Superfine (Pharmacia Biotech, Uppsala, Sweden). The system was composed of a peristaltic pump (Microperpex pump 2132, LKB, Bromma, Sweden); a six-way PTFE injection valve (Rheodyne, type 50, Interchim, France); a 0.5-ml injection loop (Daka Ware, Chicago, USA); a glass column (height=27 cm, diameter=1 cm) containing the gel swollen in citric acid solution 9 g l^{-1}; a 10 μl circulation quartz cuvette (no. 010, Hellma, Germany) and a fraction collector (Microcol, Gilson TDC80, Villiers le Bel, France).

Elution was carried out at a flow-rate of 0.05 ml min^{-1} using citric acid solution (9 g l^{-1}) as the mobile phase and 1-ml fractions were collected. Unless otherwise stated, the gel was first saturated with lipid by passing a liposome suspension without rIL-2 overnight in a closed loop. The elution of rIL-2 liposomes and free rIL-2 was monitored by measuring the fluorescence with a four-channel fluorimeter Spex F1T11I (equipped with Peltier-cooled photomultipliers and a 450 W xenon lamp) controlled by DM 3000 software (Spex Industries, Edison, USA). Fluorescence emission was measured at 90°. Samples were maintained at 25 °C by a water bath (Lauda RCS6, Lauda, Königshafen, Germany). rIL-2 elution was monitored at 320 nm (tryptophan fluorescence emission) after excitation at 290 nm, while liposomes were detected at the same time by light scattering at 290 nm.

2.5. rIL-2 SDS–PAGE

rIL-2 samples were boiled in Laemmli sample buffer [13] and run on 12% SDS–PAGE. Proteins in the gel were stained with silver nitrate according to the method described in Ref. [14] under reducing (10% β-mercaptoethanol) conditions. Bands were compared with standard molecular mass markers (Gibco, Cergy-Pontoise, France).

2.6. Quantification of rIL-2 by high-performance liquid chromatography (HPLC)

rIL-2 was assayed by HPLC according to the method of Fleury et al. [10]. A Vydac (CA, USA) C_4 butyl-silanol column (214 tp 5415, 4.6 mm×20 cm) was connected to a Waters chromatography system (Waters Millipore, France). Samples (50 μl) were injected from a Waters 717 autosampler (Waters

Millipore, France). The mobile phase was a linear gradient of two solutions A and B [A: 30% acetonitrile (Carlo Erba, Val de Reuil, France), 70% purified water; B: 70% acetonitrile, 30% purified water; both containing 0.1% trifluoroacetic acid (Sigma, St Louis, MO, USA)] from 75 to 100% A over 10 min at a flow-rate of 2 ml min^{-1}. rIL-2 was detected by UV absorbance at 280 nm; the retention time of the protein was about 11.5 min. rIL-2 associated with liposomes was determined by HPLC under the same conditions. The rIL-2 liposomes were injected onto the column directly. They were disrupted by the initial mobile phase and the solubilised DPPC was eluted early. The lower limit of detection of rIL-2 was 9 μg ml^{-1} and its calibration curve was found to be linear in the range 9–200 μg ml^{-1}.

2.7. Quantification of rIL-2 by fluorescence intensity

Fluorescence measurements were made in a 10-μl quartz circulation cuvette positioned in the spectrofluorimeter as described in Section 2.4.

2.7.1. Quantification of free rIL-2

rIL-2 was introduced by a syringe into the quartz cuvette. Sample fluorescence was excited at λ_{exc}= 290 nm and emission was recorded at a maximum at about λ_{em}=320 nm. The emission fluorescence intensity of the citric acid solution was subtracted from that of the sample. The value reported is the mean of at least three measurements.

2.7.2. Transformation of liposomes into micelles

Three milliliters of liposome suspension (2.02 or 1.01 m*M* phospholipid) were introduced into the quartz cuvette and was maintained at 25 °C in the spectrofluorimeter as described in Section 2.4. A syringe containing an OG solution (113.8 m*M*) was placed in a system which pushed the piston at a constant flow-rate of 0.007 ml min^{-1}. Liposome disruption was observed by light scattering and break points were determined by the tangents method [15].

2.7.3. Quantification of rIL-2 associated with liposomes

Liposomes (200 μl, 1:1 dilution) were disrupted and solubilised by 2.25 mg of OG. Samples were analysed with the set-up described in Section 2.7.1. The fluorescence intensity of a micellar solution (1.2 mg DPPC, 58 mg OG, 5 ml water) was used as a blank. Each result is the mean of three measurements. A calibration curve was obtained by dilution of IL-2 in the micellar solution of DPPC/OG/water.

3. Results and discussion

3.1. Analysis of free IL-2

Several papers have already described the quantification of free rIL-2 by an HPLC method [10,12]. Our rIL-2 samples contained some dextran even after purification, which modified the chromatograms (variability of retention time and baseline) (data not shown). Elimination of dextran from the column, by buffer injection, renders the technique even more time-consuming. Therefore, a new, more rapid method of analysis would be advantageous.

The rIL-2 molecule contains one tryptophan and three tyrosine residues which all possess intrinsic fluorescence that could be used to determine its concentration. The selectivity of the analysis depends on the choice of excitation wavelength. Although the tryptophan fluorescence has an excitation maximum of 295 nm, its emission spectrum is less modified by the Raman emission band of water when it is excited at 290 nm. All analyses of free rIL-2 were therefore made with an excitation wavelength of 290 nm.

When OG was added, the environment of the tryptophan changed and consequently its excitation maximum. By fixing the emission wavelength at 320 nm and varying that of excitation, the maximum fluorescence was obtained at 280 nm. This excitation wavelength was therefore used for all experiments in the presence of OG. It was necessary to check that there was no interference between dextran and IL-2 fluorescence. The IL-2 fluorescence intensity was not changed by dextran, whatever the concentration (data not shown) and no influence on its emission spectrum was observed.

Standardisation of the fluorescence method was obtained using different samples prepared on different days by two different operators. To verify the correlation coefficient, reproducibility, repeatability

and accuracy, standards and controls were analysed three and five times, respectively. The linearity was evaluated by fitting calibration curves by linear regression. The coefficient of variation (CV) of the inter- and intra-day precision and the mean accuracy were around 3 and 7%, respectively. The mean value of the correlation coefficient was 0.998 (Table 1).

Fig. 1 represents typical calibration curves for rIL-2 measurement by HPLC and fluorimetry. This latter technique yields a fluorescence value which depends on many parameters and is a relative measurement, therefore calibration is required each time the technique is used. The limit of detection was about 4.5 μg ml^{-1} and the lower limit of quantification was estimated to be about 10 μg ml^{-1}, in a experiment other than that shown in Fig. 1. All results were included within acceptable validation of FDA [16] and more than 0.950 for correlation coefficient. In conclusion, free rIL-2 analysis by fluorescence was validated.

By fitting the experimental points (x=fluorescence intensity and y=mean area of the HPLC peak for the same sample) by linear regression, a correlation coefficient of about 0.993 was obtained; that is to say, HPLC and fluorescence yielded equivalent results. The limit of detection for HPLC (5 μg ml^{-1}) was slightly higher than that of fluorescence (≤4.5 μg ml^{-1}) and the correlation coefficients were the same. It was observed that this limit of detection depended on the residual fluorescence of water. Indeed, these fluorescent impurities in the solvent could mask rIL-2 fluorescence at low concentration[1].

[1]The residual fluorescence of pure water observed above was probably due to the presence of azulene molecules. It is likely that these molecules, used as whiteness enhancers, especially in washing powder, are not completely eliminated during water treatment processes and are therefore recycled in tap water. They have already been completely observed and identified as such by mass spectrometry (M. Ollivon, unpublished results). Furthermore, we found that these molecules were not fully eliminated from water by purification using ELGA R10 or Millipore Milli-Q systems. Interestingly, this fluorescence increases when a detergent, such as OG, is added to the water. This effect is due to the entrapment of azulene by detergent micelles. The quantum yield of the azulene molecule in micelles is enhanced by the change in dielectric constant of this hydrophobic medium. This observation could be exploited to provide a quantitative measurement of azulene contamination of water samples.

Table 1
Typical data corresponding to the calibration curve and the controls

Calibration curve							
Concentration (μg/ml)	204.22	144.07	92.75	74.11	51.04	32.5	9.38
Fluorescence intensity (cps)	139 521.54	94 634.08	61 029.90	45 582.93	30 436.07	16 352.25	2972.30
	140 222.74	93 285.35	61 399.10	45 769.48	30 096.50	15 575.30	3061.97
	138 986.04	93 985.73	62 152.55	45 792.42	31 043.74	16 308.42	3099.88
Mean fluorescence intensity	139 576.77	93 968.39	61 527.181	45 714.94	30 525.44	16 078.66	3044.72
SD	620	674	572	115	480	436	65
CV (%)	0.44	0.71	0.93	0.25	1.57	2.7	2.1
Controls							
Fluorescence intensity (cps)	119 332.64	50 410.13	13 050.19				
	116 223.64	49 877.53	12 708.30				
	113 227.59	49 309.84	13 228.81				
	118 123.90	50 178.44	12 774.02				
	116 765.00	48 143.71	13 624.04				
Mean fluorescence intensity	116 734.55	49 583.93	13 077.07				
SD	2304	904	371				
CV	1.97	1.82	2.8				
Real concentration (μg/ml)	172.85	76.29	25.27				
Calculated concentration (μg/ml)	173.54	78.06	26.15				
Accuracy (%)	0.4	2.3	3.5				

Equation of the calibration curve: fluorescence intensity=(703.28×rIL-2 concentration)−5316.15. Quantification of rIL-2 by fluorimetry (λ_{ex}=290 nm; λ_{em}=320 nm).

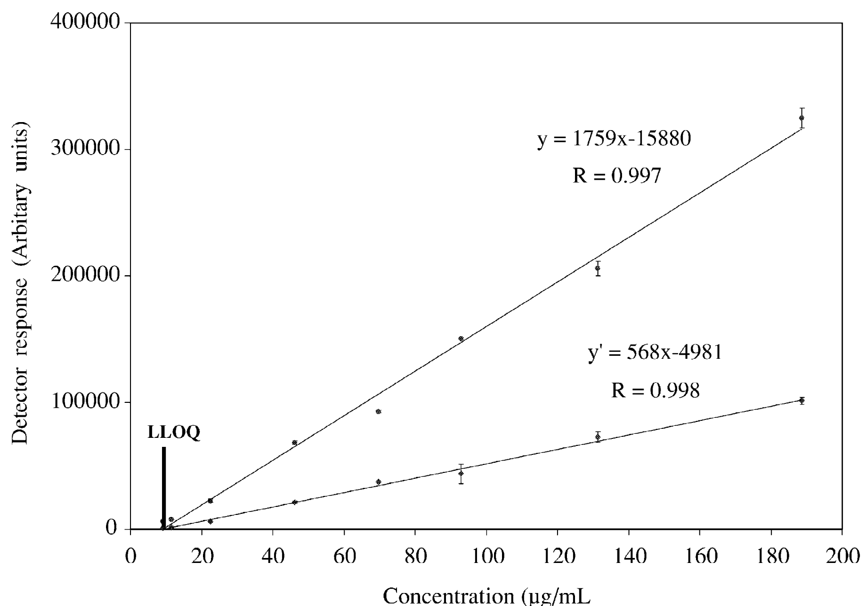

Fig. 1. Calibration curves for rIL-2 measurement by HPLC (•) and fluorimetry (♦). Samples for the two techniques were prepared from the same standard citric acid-containing mother solution obtained by rehydration of a lyophilisate. •, HPLC 280 nm UV detector response (elution rate: 2 ml/min); ♦, maximum fluorescence intensity measured at $\lambda_{em}=320$ nm. Correlation between the two sets of detector responses y and y' yields $R=0.996$. LLOQ, Lower Limit of Quantification (about 10 μg ml^{-1}).

rIL-2 analysis by fluorescence used a small amount of sample (10 μl) and was rapid (60 s per sample).

3.2. GEC methodology for separating free and associated rIL-2

In order to quantify associated rIL-2, it was necessary to separate free rIL-2 and rIL-2 associated with liposomes and to solubilise the liposomes.

GEC has often been used for separating liposomes from smaller objects. The method described here (Fig. 2) is original in that it combines on-line measurement of light scattering to monitor the elution of liposomes with detection of the protein by fluorescence. However, good resolution of free rIL-2 from liposomes requires the protein to be in its monomeric form in solution. Indeed, rIL-2 can self-associate to form dimers or aggregates [17,18] which, if sufficiently large, could co-elute with the liposomes, scatter light and/or block the mobile phase circulation through the column. Therefore, GEC was carried out at pH 3 to ensure rIL-2 solubility. Under these conditions, liposomes scatter much more light than the protein and can therefore be identified by light scattering at 90° ($\lambda_{emission}=\lambda_{excitation}$). To decrease the intensity of the light scattered from the liposomes and prevent saturation, a glass slide acting as a high-pass filter was placed in front of the emission photomultipliers.

The chromatograms obtained after elution of free rIL-2, liposomes alone and rIL-2-loaded liposomes are shown in Fig. 3. The elution profile of free rIL-2 alone (Fig. 3C) indicates that two peaks were detected by emission but only one by light scattering (however, it should be noted that the ordinate scale is very different from that of the other chromatograms in which liposomes were loaded and thus that this latter peak was small). This peak detected by light scattering corresponds in elution volume to the first peak detected by emission (8.5–17 ml). The second peak detected by fluorescence at 320 nm (17.5–26 ml) was not observed by light scattering and corresponds to free, monomeric rIL-2 (15 kDa). The elution volume of the first peak indicates that it

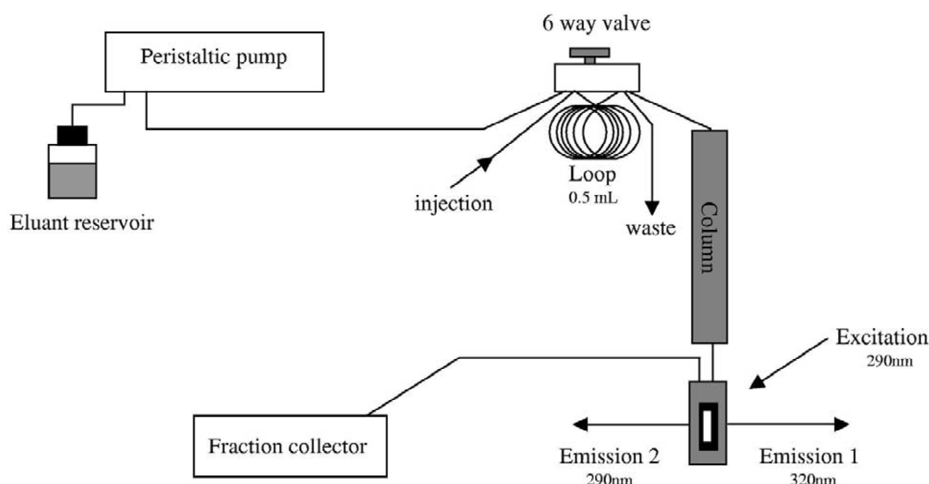

Fig. 2. Gel exclusion chromatography set-up. An eluant reservoir, a peristaltic pump, a six-way valve with syringe, loop and waste, a GEC column, a fluorimeter and a fraction collector were connected in series as shown schematically.

corresponds to much larger objects than the monomeric protein and, furthermore, it was also observed by light scattering, whereas, as explained above, rIL-2 did not diffuse light under these experimental conditions. To determine the nature of the entities generating this peak, electrophoresis followed by silver staining was performed. After GEC, the sample corresponding to this peak was too dilute to be analysed by electrophoresis, so hydrated rIL-2 from the stock vials was used. Minor bands of weaker intensity with a higher molar mass than rIL-2, were detected in the sample (Fig. 4). The presence of these bands could explain the light scattering observed in GEC. Since they were detected in the presence of SDS and β-mercaptoethanol, these bands are unlikely to correspond to aggregates of rIL-2 molecules and probably originate from bacterial proteins co-purified with the cytokine. Similar results (not shown) were obtained in the absence of β-mercaptoethanol, thus excluding the hypothesis of rIL-2 aggregates formed by intermolecular disulphide bonds, but since electrophoresis was always carried out in the presence of SDS, the possibility that non covalent aggregates of rIL-2 contributed to the first peak shown in Fig. 3C cannot be ruled out.

Liposomes without rIL-2 (Fig. 3D) were excluded from the gel at an elution volume of about 7–12 ml, lower than that of free rIL-2. A very weak peak was detected by emission at 320 nm at the same elution volume. This peak corresponds to light scattered by liposomes, which by their size are excluded from the Sephadex G75 column. The elution profiles recorded for the liposomes containing rIL-2 prepared by extrusion (Fig. 3B) or sonication (Fig. 3A) were different. The elution volume of the first peak, corresponds to that obtained with liposomes alone, and the light scattering and the fluorescence emission at 320 nm observed indicated that this peak corresponded to liposomes with associated rIL-2. Furthermore, in the case of liposomes obtained by extrusion, two peaks were detected by emission at 320 nm. The peak at 17–23 ml had no equivalent in light scattering, and the emission spectrum of the fraction corresponding to this peak was similar to that of free rIL-2 (data not shown). In conclusion, the chromatograms clearly indicate that free rIL-2 can be separated from vesicles using GEC and show that the quantity of rIL-2 associated by the sonication method is higher than that yielded by extrusion. The complete association of rIL-2 obtained by the sonication method is in agreement with results described by Fleury et al. [10]. On the other hand, when liposomes were prepared by extrusion, some non-associated rIL-2 was detected. The percentage of associa-

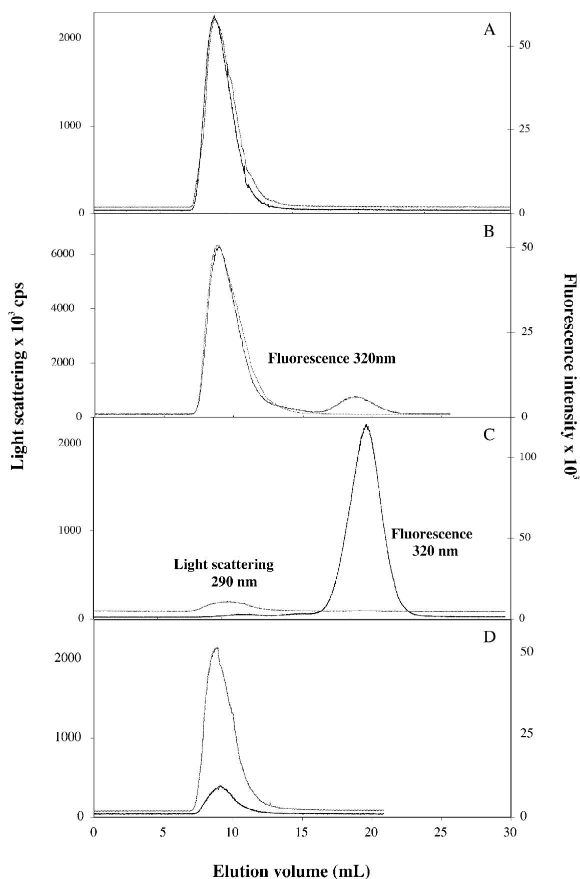

Fig. 3. Gel exclusion chromatography of free and liposome-associated rIL-2. (A) Sonicated rIL-2-containing liposomes; (B) extruded rIL-2-containing liposomes; (C) free rIL-2; (D) sonicated empty liposomes. Sephadex G75 column, Eluent: aqueous buffer (citric acid 9 g l^{-1}); sample loading: 500 μl; flow-rate: 0.05 ml min^{-1}; detection: left-hand ordinate 90° light scattering at 290 nm; right-hand ordinate fluorescence (the excitation and emission wavelengths were 290 and 320 nm, respectively).

tion, calculated by comparison of peak heights, was found to be about 90%, which was acceptable for clinical applications.

3.3. Solubilisation of rIL-2-containing liposomes

Although the presence of rIL-2 in the liposome peak could be detected by fluorescence emission, in order to quantify it, the vesicles had to be dissolved in order to avoid interference of light scattering with fluorescence measurements. This was achieved by addition of OG, a tensioactive agent which can disrupt liposomes under appropriate conditions. The transformation of DPPC vesicles into mixed micelles with OG has already been studied and the monitoring of turbidity during OG addition has shown two characteristic points at different stages of the solubilisation process [15].

Fleury et al. [10] demonstrated that the interactions between phospholipid molecules were reduced when rIL-2 was associated with liposomes, suggesting that the protein was inserted into the membrane. Since this could modify the conditions of liposome solubilisation, the disruption of rIL-2-containing liposomes under the same conditions as described previously [15] was investigated. Solubilisation was monitored by light scattering; since micelles are much smaller than both liposomes and the wavelength of light, their scattering is much less than that of liposomes and the transition can be detected easily.

Two different concentrations of rIL-2 liposomes were studied. The solubilisation profiles were divided into three parts with characteristic points determined by the tangents method (Fig. 5). In previous work, it was observed that the size of DPPC liposome first decreased to reach a minimum, then increased and decreased again until a clear micellar phase was obtained [15]. In the case of rIL-2 liposomes, their size decreased to a minimum corresponding to the micellar phase (Fig. 5).

Dahim [15] has described an equation representing the relationship between the total concentration of OG and the lipid concentration:

$$[\mathrm{OG}]_{\mathrm{tot}} = [\mathrm{OG}]_{\mathrm{solution}} + \mathrm{Rag}[\mathrm{DPPC}]$$

$$\mathrm{Rag} = [\mathrm{OG}]_{\mathrm{agg.}}/[\mathrm{DPPC}]$$

with $[\mathrm{OG}]_{\mathrm{solution}} = 15.5$ m*M* and Rag = 2.07.

Meyer [19] showed the OG distribution in the presence of protein can be written as:

$$[\mathrm{OG}]_{\mathrm{tot}} = [\mathrm{OG}]_{\mathrm{solution}} + \mathrm{Rag}[\mathrm{DPPC}] + \mathrm{Re}[\mathrm{Protein}]$$

$$\mathrm{Re} = [\mathrm{OG}]_{\mathrm{agg.}}/[\mathrm{Protein}]$$

We assume that the ratio between OG and DPPC concentrations in the final aggregates (Rag) is the same in the presence or absence of rIL-2. To

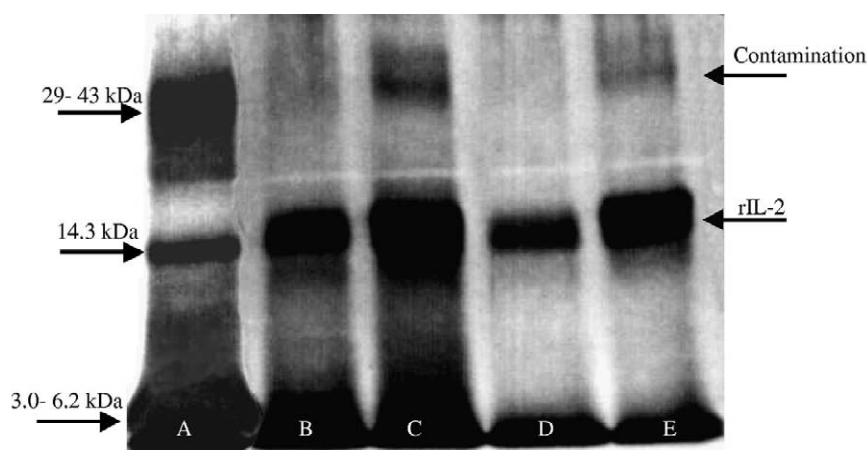

Fig. 4. Silver staining of rIL-2 in SDS–polyacrylamide gels (under reducing conditions as described in Ref. [12]). (B–E) rehydrated rIL-2 lyophilisate; (A) low molecular mass range protein standards; (C) undiluted; (B) diluted 1:10; (D) diluted 1:100; (E) undiluted after purification to remove dextran.

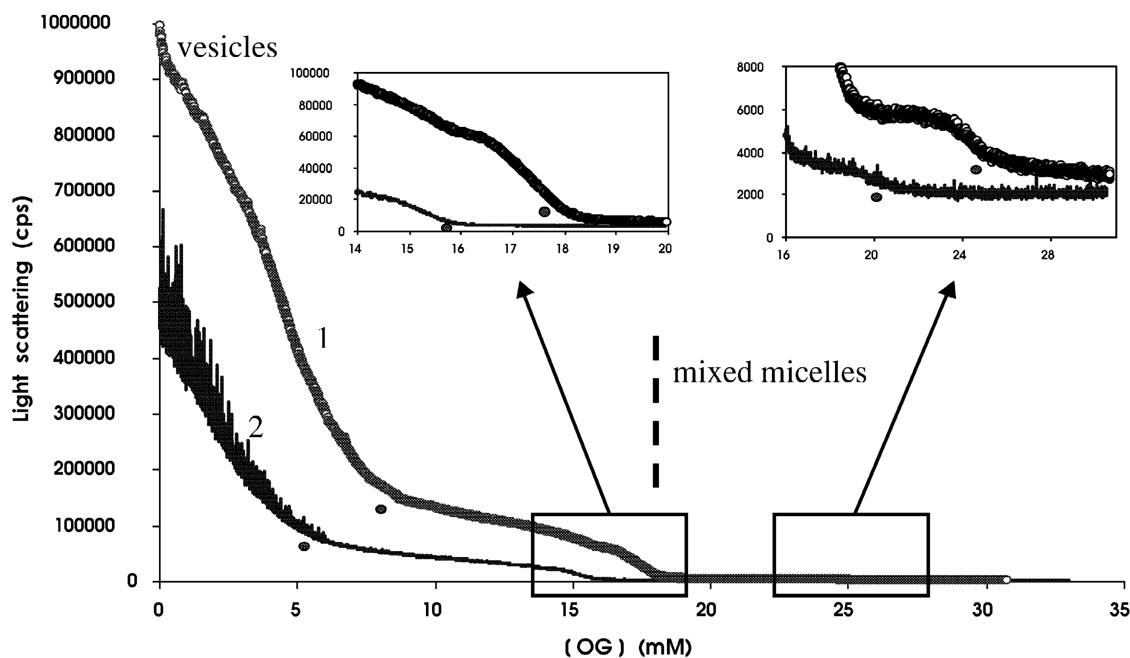

Fig. 5. Solubilisation profiles for DPPC liposomes containing rIL-2. OG solution: 113.8 mM; OG addition rate: 0.007 ml min^{-1}; detection: 90° light scattering at 290 nm; initial sample loading: 3 ml of DPPC suspension associated with rIL-2; (1) 2.02 mM DPPC—111 μg ml^{-1} rIL-2; (2) 1.01 mM DPPC—56 μg ml^{-1} rIL-2. •, break points.

solubilise rIL-2 liposomes containing 1.02 mM and 2.02 mM DPPC, respectively, 21.6 and 26.1 mM OG were necessary. By using the relationship above, we could calculate $[OG]_{solution} = 16.5$ mM and Re = 0.073 mmol μg^{-1}.

Therefore, DPPC–OG interactions are modified by rIL-2, since the OG concentration necessary for complete solubilisation is increased in the presence of the protein. rIL-2 seems to stabilise the phospholipid bilayer, but the nature of this interaction remains to be investigated. In order to completely solubilise liposomes by 30 mM of OG, it was very important to ensure that the DPPC concentration after dilution of samples was less than 2 mM.

3.4. Influence of OG on rIL-2 fluorescence

Changes in conformation of the IL-2 molecule have been observed to modify the tryptophan fluorescence spectrum as a result of the changing environment of this amino acid [20,21]. OG could interact with IL-2 and unfold the protein by making mixed micelle-protein aggregates and thus affect the fluorescence profile, as already described for a viral envelope protein [22]. Indeed, the emission spectra of rIL-2 varied with OG concentration until liposome solubilisation was obtained at about 30 mM (Fig. 6). After complete solubilisation, the addition of excess OG did not modify the fluorescence spectrum of rIL-2. Furthermore, as specific interaction of phospholipids with rIL-2 or residual fluorescence of these lipids cannot be ruled out, it is necessary to establish calibration curves in the presence of similar amounts of the lipids used to prepare the liposomes.

3.5. Quantification of rIL-2 associated with liposomes by fluorescence

A calibration curve for the fluorescence assay was obtained by dilution of rIL-2 in a micellar solution of DPPC/OG. The CV of the inter- and intra-day precision and accuracy were all 2.0%. The mean value of the correlation coefficient was 0.998. The limit of detection was variable, about 6.0–7.0 μg ml^{-1} and the lower limit of quantification was

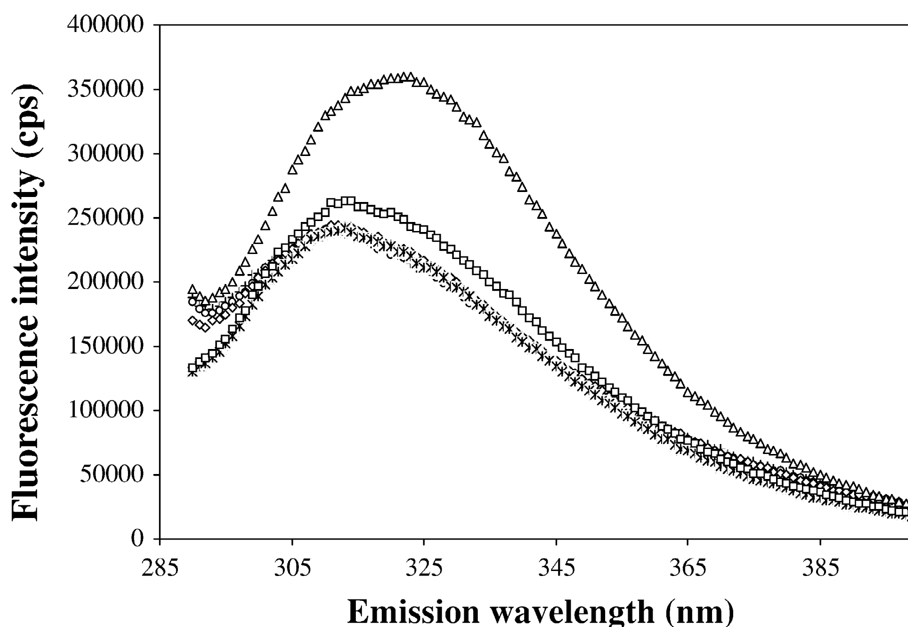

Fig. 6. rIL-2 emission spectra in the presence of increasing concentrations of OG. The excitation wavelength was 280 nm. OG concentrations: △, 13 mM; □, 20 mM; *, 40 mM; ◊, 56 mM + 69 mM; ○, 81 mM.

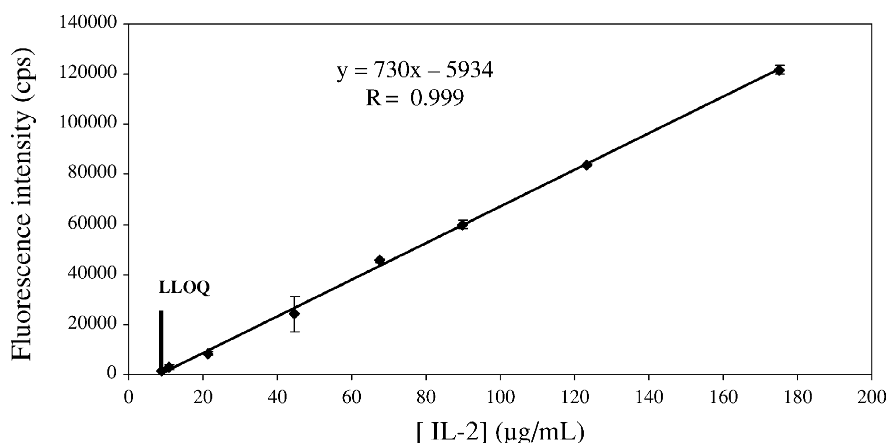

Fig. 7. Calibration curve for fluorimetry detection for rIL-2 released from vesicles. Samples were obtained from the standard citric acid-containing mother solution obtained by lyophilisate rehydration diluted in a micellar solution (1.2 mg DPPC/58 mg OG/5 ml water). The excitation and emission wavelengths were 280 and 320 nm, respectively. LLOQ: Lower Limit of Quantification (10 μg ml^{-1}).

estimated to be about 10.0 μg ml^{-1} (Fig. 7). All these results were included within acceptable validation limits of FDA [16]. In conclusion, rIL-2 liposome quantification by fluorescence was possible and has been validated.

In this study, the precise determination of the concentration of associated rIL-2 concentration was possible in the range of 10.0–200 μg ml^{-1} after separation by GEC and solubilisation by OG.

4. Conclusion

This study describes the development of a quick and accurate fluorescence method for the evaluation of rIL-2 in liposome preparations, which requires only small IL-2 samples. A continuous system, which would separate by GEC and identify the liposomes by light scattering can be envisaged. An injection system will introduce OG after liposome detection, then rIL-2 will be quantified by fluorescence.

We have also shown that a large fraction of the rIL-2 present in the hydrating solution is associated with liposomes. The efficiency and the sustained release properties of this system will be evaluated for future use in immunotherapy and as an immunological adjuvant.

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