MULTI-FLUORESCEIN-SUBSTITUTED POLYMERS AS POTENTIAL LABELS IN FLUOROIMMUNOASSAY

A system for improved detection sensitivity

Mithal HASSAN, J. LANDON* and D. S. SMITH

*Technia Diagnostics Ltd, 48, Bartholomew Close, London EC1A 7HL, and *Department of Chemical Pathology, St Bartholomew's Hospital, 51, Bartholomew Close, London EC1A 7HL, England*

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1. Introduction

Fluoroimmunoassay (FIA) provides a versatile alternative to radioimmunoassay (RIA), but its applications are restricted by its relatively lower sensitivity [1,2]. In principle, FIA sensitivity could be improved by attaching many fluorescent groups to the labelled reagent [3,4]. Polyamines such as poly-lysine and polyethyleneimine, which are available in degrees of polymerisation up to several hundred, are suitable for simple reaction with fluorescein isothiocyanate (FITC) to provide multi-fluorophore labels [3]. Unfortunately fluorescein, which is the label of choice and the most widely used in conventional FIA [1,2], is susceptible to 'concentration quenching' in multi-substituted polymers [3] or proteins [5,6], such that increased incorporation of fluorescent groups soon becomes self-defeating.

We tested a method for the indirect expression of the inherent potential fluorescence of a multi-fluorescein-substituted polymer which involves the use of antibodies to fluorescein and exploits the well-known quenching of the fluorescence of fluorescein groups bound by specific antibody [7,8]. The system is illustrated schematically in fig.1.

2. Experimental

FITC isomer I and poly(L-lysine) (PL) hydrobromide (degree of polymerisation 150) were from Sigma, and fluorescein sodium salt from Koch-Light. Fluoresceinthiocarbamyl gentamicin (FTC-G) [9] and rabbit anti-fluorescein serum [10] were prepared as described.

FITC (2 g/l; 5 ml) and PL hydrobromide (6.7 g/l; 2 ml) were each dissolved in 10 ml/l aqueous triethylamine. The solutions were mixed (molar ratio FITC:PL 60:1) and reaction allowed to proceed overnight at room temperature. Chromatography on Whatman no. 3MM paper developed with sodium bicarbonate buffer (50 mM, pH 9.0) indicated almost complete reaction (PL was visualised by spraying with 3 g/l ninhydrin in methanol). R_F values were: PL, 0.98; FITC 0.43; the product (multi-FTC-PL) 0.0. The product was precipitated by addition of 28 ml ammonium acetate buffer (50 mM, pH 5.0), collected by centrifugation, washed twice with the same buffer, and freeze-dried.

Fluorescein concentrations were estimated using the extinction coefficient in [11], and those of FTC-G by assuming it to have the same extinction as fluorescein at 492 nm in 50 mM sodium bicarbonate buffer (pH 9.0). Concentrations of multi-FTC-PL were estimated by weight, assuming complete incorporation of 60 fluorescein groups/polymer molecule.

All fluorescence experiments were performed at room temperature using 100 mM sodium phosphate buffer, (pH 7.5) containing 1 ml/l Triton X-100 detergent. Fluorescence was measured using a Perkin-Elmer Model 1000 fluorimeter equipped with filters as described [12]. Intensities were expressed on a

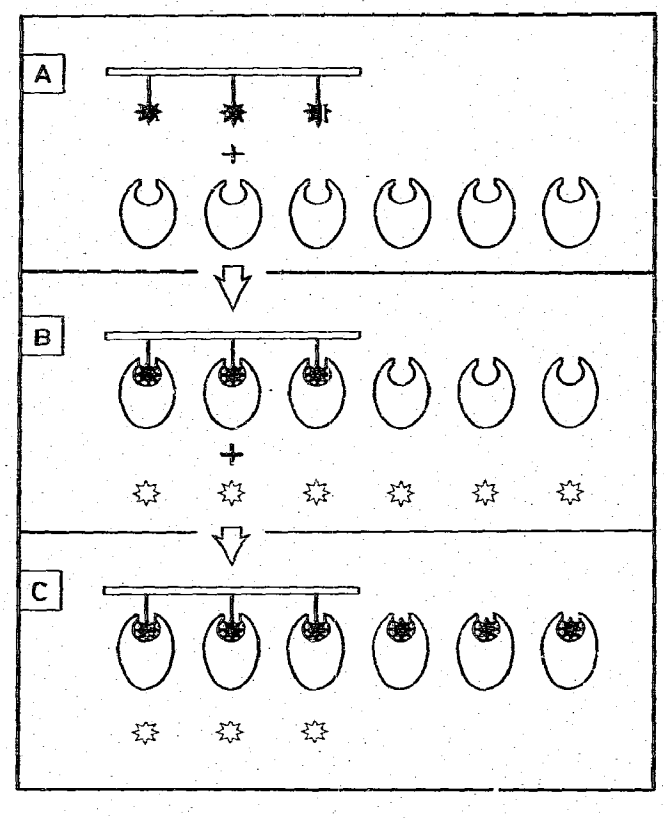

Fig.1. Method for detection with improved sensitivity of a multi-fluorescein-substituted polymer (A) in which the fluorophores are subject to concentration quenching. First an excess of antibodies to fluorescein is added and allowed to bind the fluorescein groups of the polymeric reagent (B). Next, the remaining unoccupied antibody sites are 'back-titrated' by addition of a monomeric fluorescein reagent which is free to fluoresce with high yield. Those monomeric fluorophores which become antibody-bound are efficiently quenched, while the remainder give a fluorescence signal which is directly related to the initial amount of polymer, but enhanced in relative magnitude (C).

standardised scale defined such that 1 nM fluorescein gave a signal of one unit.

To test the proposed system, doubling dilutions of multi-FTC-PL were prepared and a 500 μl aliquot of each was mixed with 500 μl 6000-fold diluted anti-fluorescein serum. After 30 min, 500 μl 30 mM FTC-G was added to each mixture, and after a further 30 min fluorescence was measured. The signals from doubling dilutions of multi-FTC-PL alone and of FTC-G alone were also measured.

3. Results

An antibody dilution curve showed that the fluorescence of 10 mM FTC-G was quenched from 5.8 units to 0.42 units in presence of excess anti-fluorescein serum.. A final antiserum dilution of 18 000-fold, which quenched FTC-G fluorescence to 0.81 units, was chosen for use in the detection system.

At final concentration 50–750 pM the fluorescence signal indirectly generated from multi-FTC-PL using the detection system was 9–11-times greater than that obtained from FTC-G by conventional fluorimetry (fig.2). The fluorescence signals from multi-FTC-PL and FTC-G alone were 82% and 58%, respectively, of that of fluorescein at the same concentration.

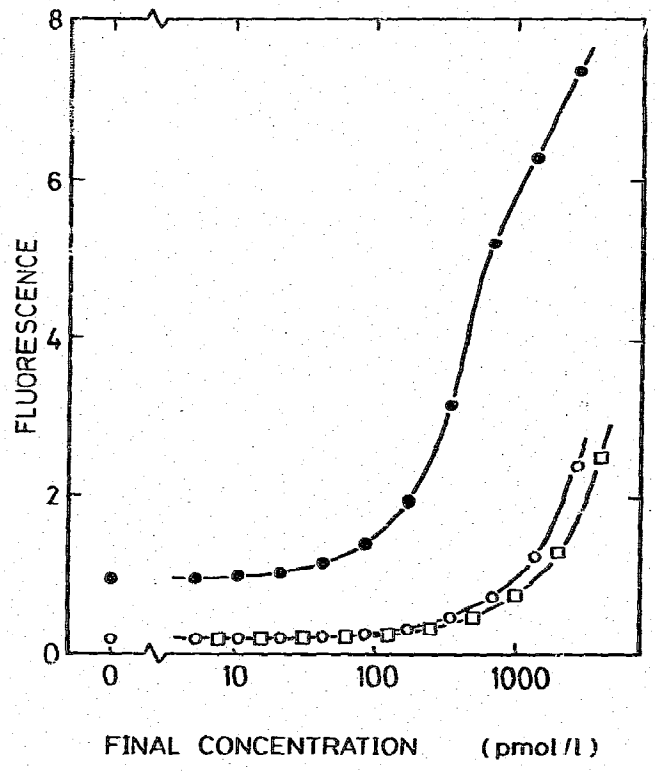

Fig.2. Fluorimetric detection of multi-FTC-PL using anti-fluorescein serum and FTC-G (closed circles) compared with that of multi-FTC-PL alone (open circles) and FTC-G alone (open squares). Total fluorescence signals are shown; the buffer background signal was 0.17 units.

4. Discussion

An elegant solution to the problem of concentration quenching of fluorescein was proposed and demonstrated [3]. In 'quantum yield-independent fluorimetry' the sample is illuminated to complete photochemical bleaching using a powerful laser, with the result that each fluorophore emits the maximum possible number of photons, a quantity shown to be independent of the conventional quantum yield. The method has been used to detect single antibody molecules labelled with multi-fluorescein-substituted polyethyleneimine [13]. However, the instrumentation involved is complex and costly.

An alternative approach is the use of a fluorophore which is not subject to concentration quenching [4]. Multi-umbelliferone-substituted polylysine labels were used in steroid FIA methods which showed sensitivity equal to that of equivalent RIA systems employing tritiated ligands [4]. The umbelliferone fluorophore shows no concentration quenching at the level of ~ 1 fluorescent group/10 lysine residues, as judged by the agreement between fluorimetric and photometric assessment of the degree of substitution [14]. However, the preparation of a suitable umbelliferone derivative and its conjugation to the polymer is more involved than simple reaction with FITC.

These studies have shown the feasibility of a system for improved detection of multi-fluorescein-substituted polymers which involves easily-prepared reagents used in aqueous buffer at room temperature and requires no specialised instrumentation. FTC-G was a convenient choice as the monomeric fluorescein reagent because, unlike fluorescein and most fluorescein-labelled small molecules, it does not become non-specifically bound and quenched by serum proteins [9].

Over a 15-fold concentration range, corresponding to the presence within the total illuminated volume of the sample solution (~ 300 μl) of some 15–225 fmol multi-FTC-PL, ~ 10-times more signal was produced from the polymer using the detection system as compared with the fluorescence of a mono-fluorescein-labelled species such as FTC-G. The extent of this useful working range would be adequate for the intended application, since immunoassay standard curves generally cover a < 10-fold variation in labelled reagent concentration.

Optimisation of the detection system might include a study of the effects of polymer size and degree of fluorescein-substitution; choice of conditions to allow the use of fluorescein itself as the monomeric reagent (almost twice the signal obtained using FTC-G would be expected); and reduction in incubation times (reactions between fluorescein groups and specific antibodies are complete within a few minutes at concentrations similar to those used in these studies [10]).

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