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Fluorometric method for the simultaneous quantitation of differently-sized nanoparticles in rodent tissue☆

Nasir Hussain *

Centre for Drug Delivery Research, 29–39 Brunswick Square, The School of Pharmacy, University of London, London WC1N 1AX, UK

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Abstract

The oral absorption and systemic translocation of particulate matter via the gastrointestinal tract has been shown by a number of laboratories using a wide variety of particles in different animal species. While there is debate on the magnitude of particle intestinal translocation, which is encumbered by the differing experimental protocols, particularly the method of quantitation of absorbed material, few have sought to examine the pharmacokinetic aspects of particle absorption. We describe in this communication the development of a simple and a rapid fluorometric assay of quantifying tissue-laden fluorescent nanoparticles that is able to isolate, detect and quantify the presence of two or more particle populations differing both in their size and fluorescent label. Six types of polystyrene nanoparticles incorporating different fluorescent markers were spiked in whole livers. The fluorophores were extracted using our previously developed method of freeze-drying the tissue and using chloroform as the extractive solvent. Only two types of particle populations, orange-labelled 40 nm and Fluoroscein-emitting 500 nm nanoparticles, were sufficiently recoverable and provided a high signal-to-noise ratio for further work. The amount of tissue and type of biological tissue type also impacted on the nanoparticle recovery and detection, reflecting, perhaps, the quenching effects of interacting tissue-derived molecules. In addition, the results also indicate that the use of nanoparticles incorporating fluorescent dyes that have emission over 500 nm overcome the tissue interfering autofluorescence for low doses of nanoparticles. The use of this fluorometric method has several advantages compared with other modes of quantitation in that it is rapid, non-radioactive and the marker is non-leaching. More importantly, it allows the simultaneous detection of multiple fluorophores such that two or more different fluorescent particle populations can be detected in the same sample. This may enable the uncharted area of pharmacokinetic parameters, such as the impedance, augmentation or site of gut uptake of differently sized particles to be studied.

Keywords: Nanoparticles; Fluorescence; Oral; Polydispersity; Quantitation

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* Tel.: +44-20-77535943; fax: +4-20-77535960.

E-mail address: nasir.hussain@cua.ulsop.ac.uk (N. Hussain).

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1. Introduction

Intestinal absorption of nanoparticles and their subsequent appearance in the systemic circulation has been proven by a number of investigators from different laboratories during recent years (Florence, 1997). A wide variety of particulate material has been shown to be absorbed and this has naturally entailed the use of specific methods of quantifying the deposition of material in the intestinal tissues and systemic organs. Radiolabelling either the carrier or encapsulated tracer, optical and mechanical counting or quantitation of tissue-extracted polymer derived from the nanoparticle material have been popular methods of detecting and quantifying organ-deposited polymeric material (Delie, 1998). Despite the variation in figures for nanoparticle absorption, which is dependent on mode of quantification and also on the experimental protocol, there is a paucity of studies devoted to the mechanistic and pharmacokinetic aspects of intestinal particulate transit and translocation. These have often been conducted with monosized nanoparticles and/or single mode of quantitation. To date, there has been no study that has investigated the simultaneous intestinal absorption of differently sized nano-/microparticles, apart from that of Eldridge et al. (1990), who quantified a population of polydisperse poly-lactide-co-glycolide particle absorption in mice by optical counting, a method prone to operator error. Given the current methods of biodegradable nanoparticle production that typically yield a diverse particle size population, the debate on the upper size limit of particle absorption and the increasing interest in the area of vaccination by the oral route using biodegradable polymers, it is pertinent to develop a method of quantifying the intestinal absorption of nanoparticles of varying size. In this regard, we present in this communication some preliminary in vitro data that utilize fluorometric techniques, often used in blood flow studies (Deveci and Eggington, 1999), to investigate some parameters involved in the simultaneous quantification of nanoparticles, of different size and fluorometric label, from spiked rodent tissue.

2. Experimental

2.1. Materials

Fluoroscein isothiocyanate-labelled 500 nm nanoparticles (2.5% w/v) were obtained from Polysciences (Warrington, UK). Nanoparticles (40 nm; 5% w/v) labelled with yellow–green, orange, lilac, red and infa-red dyes were obtained as a kit from Molecular Probes (Amsterdam, Netherlands). Chloroform (Analar grade) was purchased from BDH (UK), and Cellusolve and 2-(2-ethoxyethoxy) ethyl acetate were obtained from Lancaster chemicals. Animal tissues were provided by the School of Pharmacy's animal unit from an inbred colony of Sprague–Dawley rats.

2.2. Methods

A known of amount of nanoparticles was spiked to either macerated liver (2.5–9.5 g) or spleen (0.7 g) and freeze-dried overnight. The cake was ground with a pestle and mortar and 20 ml extractive solution added. Nanoparticle extraction was conducted in glass vessels and incubated at 30°C overnight to facilitate polymer extraction. The suspension was filtered using a Buchner funnel and a 1 ml aliquot measured via a Perkin-Elmer fluorometer (LB 50) using appropriate excitation/emission wavelengths. Nanoparticles in the absence of any animal tissues, or tissues alone and subjected to the same extraction procedures, were used as controls. Detection and efficiency of recovery of tissue-extracted flurophores was quantified using pure suspensions of freeze-dried nanoparticles.

3. Results

3.1. Efficiency of extraction solutions

Cellusolve and ethyl acetate derivatives have been reported as suitable extractive solvents for fluorescent dyes. While these solvents are suitable for extracting fluorophores from pure suspensions of nanoparticles, they are poor penetrants of animal tissues, possibly due to their polar nature.

Readings were barely above background. In contrast, chloroform is an excellent extractor and was subsequently used throughout rest of the experiments (data not shown).

3.2. Detection of polymer-incorporated fluorophore in the presence of tissue

Six different fluorophores with varying excitation/emission wavelengths were investigated for their ability to discriminate fluorophore signals from background tissue autofluorescense. Two hundred micrograms of polystyrene nanoparticles were spiked in 2.5 g liver tissue. Table 1 shows the normalized signal-to-noise ratio of each of these flurophores after dividing by the autofluorescent signal from blank liver. It is seen that orange- and red-labelled nanoparticles give the best signal-to-noise ratio. This can be explained by Fig. 1, which displays the excitation/emission scan of a blank liver sample. It is seen that emission of extracted material from a blank liver falls considerably above an excitation wavelength of 520 nm, hence intensifying a flurophore emission signal of a dye to be detected in this region. However, FITC-labelled nanoparticles in addition to the orange-dyed nanoparticles were subsequently used as the absolute signal of red/far-red-labelled nanoparticles was low, which would reduce its sensitivity to lower doses, i.e. 50 μg or less (Fig. 2). This is due to the limitation of the photomultiplier tube in the fluorometer that cannot sufficiently amplify emission signals above 550 nm. Yellow–green-labelled particles, despite their emission being below that of threshold, could not be used as it was impossible to discriminate signals between a blank liver sample and spiked tissue, presumably due to the short (10 nm) Stoke's shift and its emission wavelength overlapping that of the of the liver (Table 2).

Table 1
Normalized signal-to-noise (autofluorescence) ratio of each fluorophore in the presence of liver tissue

Fluorophore	Excitation	Emission	Signal/noise ratio
FITC	458	540	7
Yellow–green	505	515	1
Orange	540	560	18
Red	580	605	11
Far-red	660	680	1.5

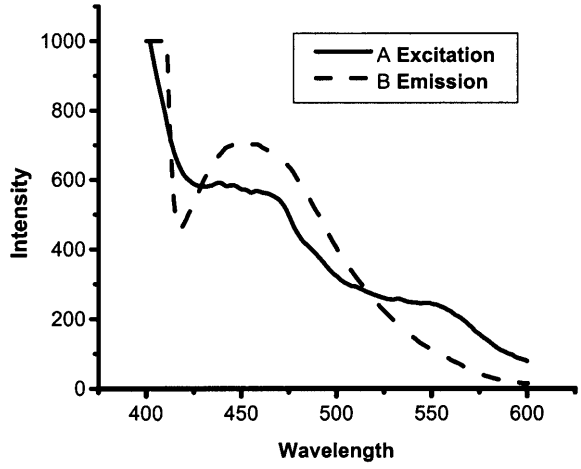

Fig. 1. Excitation and emission scan of a blank liver sample (no nanoparticles). Macerated liver was freeze-dried and the lyophilized cake suspended in 20 ml chloroform. After overnight extraction, the material was filtered and 1.4 ml filtrate sample was fluorometrically scanned for detection of maximum absorbing and emitting peaks. Maximum emission is around 472 nm with considerable tailing off after 520 nm.

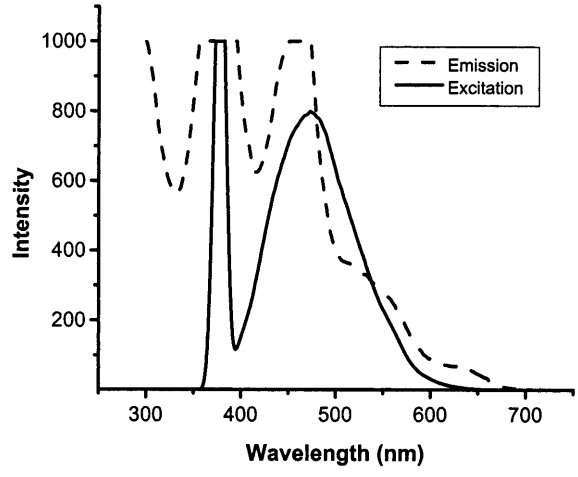

Fig. 2. Excitation and emission of a liver sample in the presence of FITC-labelled 500 nm nanoparticles. The sample was prepared as described in Section 2 and Fig. 1. The FITC emission signal is around 550 nm, its peak being obscured by the emission tail from liver-derived fluorophores.

Table 2
Relative fluorescent signals obtained from 200 μg polystyrene spiked liver tissue ($n = 8$)

Fluorophore (nm)	Excitation/emmission wavelength	Blank liver (relative fluorescent units)	Blank liver + fluorophore (relative fluorescent units)
FITC 500	458/540	103	723
Yellow–green 40	505/515	>1000	>1000
Orange 40	540/560	46	>842
Red 40	580/605	4.2	46
Far-red 40	660/680	0.15	1

3.3. Lowest limit of fluorophore detection

Intestinal absorption of nanoparticles is inversely proportional to the hydrodynamic size of the particles; surface attachment of enterocyte-internalizing ligands can significantly enhance absorption of similarly sized nanoparticles (Hussain et al., 1997; Hussain and Florence 1998). According to our previous data, quantified using gel permeation chromatography, we estimate that unmodified fluorescent polystyrene nanoparticles of 500 nm size are absorbed at the rate of 30 μg/24 h over a 10-day period, assuming linear kinetics of absorption (Jani et al., 1990). Thus, livers were spiked with 25–200 μg polystyrene. The lowest limits of detection of 40 nm orange and 500 nm FITC-labelled nanoparticles from spiked liver (9.5 g liver sample; 200 g rat) were both around 25 μg polystyrene by weight.

3.4. Effect of tissue size and type on the recovery of nanoparticles

Liver was used a test sample because it is the most complex organ in terms of the numerous molecules it contains as possible sources of interfering fluorophores. Fluorescence signals are dependent not only on the species of the fluorophore, but on the absolute amount of such fluorescent-emitting molecules. Hence, two different weights of liver samples were spiked with FITC-labelled nanoparticles. Using 2.5 g liver and whole spleen (0.8 g), recovery of nanoparticles, based on fluorescence signal, was calculated to be 65 and 77%, respectively. However, increasing the liver sample weight to 9.5 g, or whole liver from a 200 g rat, reduced efficiency of recovery to 25%. Table 3 shows that varying the amount of spiked dose to a fixed weight of liver doses not alter the efficiency of polystyrene recovery appreciably, indicating that some liver-derived component is sequestrating either the polystyrene or its covalently attached fluorophore. Pure FITC-labelled nanoparticles subjected to the same extraction for tissue-laden nanoparticles yielded a 90% recovery, further suggesting the capacity of liver to absorb/quench FITC-labelled species. Orange-labelled nanoparticles with or without liver tissue, in contrast, gave only a 4–6% recovery, indicating that this fluorophore suffered severe quenching by the chloroform solvent (Fig. 3).

3.5. Detection and quantification of multiple fluorescent-labelled nanoparticles

The aim of the study was to investigate the ability of the extractive method to isolate and identify two or more nanoparticle populations labelled with different flurophores. Hence, whole liver was spiked with 100 μg each of FITC-labelled (500 nm) and orange-labelled (40 nm) particles. Table 4 demonstrates that both species

Table 3
Recovery of varying doses of FITC 500 nm nanoparticles from whole liver ($n = 8$)

Amount spiked (μg)	Efficiency of recovery (%)
30	21
60	25
100	31
200	26

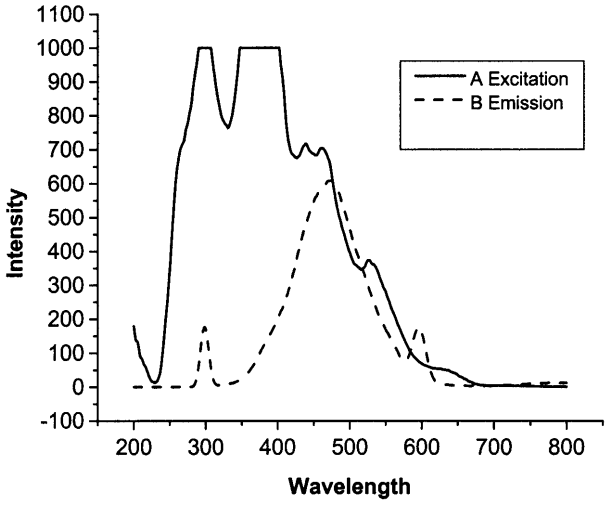

Fig. 3. Excitation and emission of a liver sample in the presence of orange-emitting 40 nm nanoparticles. Unlike FITC nanoparticles, the emission peak is clearly visible, albeit slightly shifted from that predicted for the pure fluorophore. This enables good differentiation from the interfering liver autofluorescence.

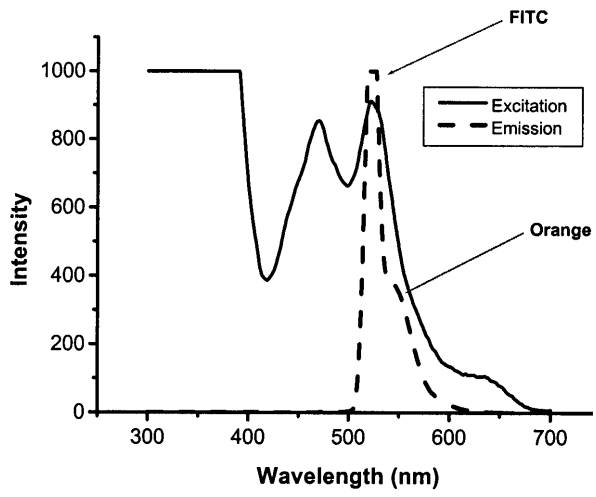

Fig. 4. A composite excitation and emission scan of a liver sample containing 500 nm FITC-labelled and 40 nm orange-emitting nanoparticles. The sample was prepared as described previously. Both peaks, as indicated by the arrows, are well separated and distinguishable from the emission peak of the liver-derived fluorophores.

could be detected easily with little interference at either excitation/emission wavelengths. Fig. 4 shows a composite scan of the same liver sample spiked with both types of nanoparticles, with the peaks of emission from both FITC- and orange-derived nanoparticles well separated from each other and from the peak emission of the liver material.

Table 4
Detection of orange and FITC fluorophores from the same liver sample ($n = 8$)

Sample	FITC (458 nm/540 nm)	Orange (540 nm/560 nm)
Blank liver	164	29
Liver + 100 μg orange	153	223
Liver + 100 μg FITC	567	54
Liver + orange + FITC	505	172

4. Discussion

While the uptake of particulate matter across the gastrointestinal tract (GIT) has been documented for over 150 years in a wide range of species ranging from the salmon (O'Donnell et al., 1996) to the dog (Wells et al., 1988), very few quantitative studies exist on the extent of particulate absorption (early studies reviewed in Verzar, 1936). Of these, there are few in the literature that document any pharmacokinetic aspects of particle translocation other than those that have investigated transport mechanistic studies using monosized particles (Wells et al., 1988; Pappo and Ermak, 1989).

It is not known whether the gastrointestinal transit, binding, cellular translocation and subsequent systemic transport of micro-/nanoparticles is a mutually exclusive event or whether a polydisperse population of particles can be internalized by the same enterocyte in a single inclusive manner. Furthermore, no information exists whether the uptake of larger particles by the enterocytes impedes the absorption of smaller particles or vice versa, due to the possible saturatability of trans-

port pathways or energy expenditure/exhaustion by the enterocyte. Thus, in this study, we have sought to investigate these parameters, prior to animal work, by developing a fluorescent-based method of isolating and quantifying two or more populations of nanoparticles differing in both their hydrodynamic size and incorporated flurophore. From Fig. 4, it can be seen that both sets of nanoparticles can be detected, with their emission peaks well separated from the maximum emission peak of liver derived material (472 nm). However, extracted FITC-labelled 500 nm nanoparticles (Polysciences) from spiked liver tissue were more easily recoverable (25–30%) compared with orange-labelled 40 nm nanoparticles (4%). This cannot be simply ascribed to the amount of fluorophore, as pure suspensions of both nanoparticles subjected to the extractive conditions, employing chloroform as the solvent and a temperature of 30°C, resulted in only a 6% recovery of orange-emitting flurophore (Molecular Probes) as opposed to $<90\%$ for FITC, suggesting that the former was drastically quenched by the solvent while the latter was affected by the liver-derived material. This is further supported by the observation that FITC-labelled material was more easily recoverable from smaller samples of added tissue (e.g. spleen, 75%). Due to proprietary concerns, the nature of the orange dye is not known, nor is weather it is covalently bound to the polystyrene in contrast to FITC-labelled nanoparticles where the dye is coupled to the polymer. Solvent-quenching effects have been noticed for other fluorophores. Despite this, the extraction of fluorophores for quantifying micro-/nanoparticles is used routinely in blood flow studies (Deveci and Eggington, 1999) and has been successfully reported, mainly using FITC as the marker, for studying GIT transport of nanoparticles in whole animals (Jani et al., 1996; Demoy et al., 1999) or with Caco-2 cells (Russell-Jones et al., 1999). The extraction method described differs from that used in blood flow studies where particles are collected from potassium hydroxide digested tissues and the dyes extracted using solvents such as Cellusolve and ethoxyethyl acetate derivatives (Akyurekli et al., 1995; Deveci and Eggington, 1999). Our attempts to use this method resulted in a severe loss of nanoparticles as, because of their lower densities, they could not be pelleted upon centrifugation. In addition, the aforementioned solvents penetrated biological tissues very poorly.

In conclusion, the preliminary data presented in this paper offer the possibility of utilizing this method, or a variant thereof, of investigating some of the aforementioned parameters. It may be further extended, for example, to compare the efficiency of two or more ligands, surface attached to nanoparticles, in promoting the uptake their uptake by the GIT, or altering the site of uptake in the same animal. Further work is in progress using fluorescent nanoparticles from different sources to isolate flurophores, yielding optimum parameters of recovery and minimizing quenching interactions due to the solvent and/or binding to tissue-derived material.

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References

Akyurekli, D.U., Gerig, L.H., Raaphorst, G.P., 1995. Tissue preparation technique for microsphere assays of blood flow. Lab. Anim. Sci. 45, 415–419.

Delie, F., 1998. Evaluation of nano- and microparticle uptake by the gastrointestinal tract. Adv. Drug Del. Rev. 34, 221–233.

Demoy, M., Andreux, J.-P., Weingarten, C., Gouritin, B., Guilloux, V., Couvreur, P., 1999. Spleen capture of nanoparticles: influence of animal species and surface characteristics. Pharm. Sci. 16, 37–41.

Deveci, D., Eggington, S., 1999. Development of the fluorescent microsphere technique for quantifying regional blood flow in small mammals. Exp. Physiol. 84, 615–630.

Eldridge, J.H., Hammond, C.J., Muelbroek, J.A., 1990. Controlled vaccine release in the gut-associated lymphoid tissue: orally administered biodegradable microspheres target the Peyer's patches. J. Control. Release 11, 205–214.

Florence, A.T., 1997. The oral absorption of micro- and nanoparticulates: neither exceptional nor unusual. Pharm. Res. 14, 259–266.

Hussain, N., Florence, A.T., 1998. Utilizing bacterial mechanisms of epithelial cell entry: invasin-induced oral uptake of latex nanoparticles. Pharm. Res. 15, 153–156.

Hussain, N., Jani, P.U., Florence, A.T., 1997. Enhanced oral uptake of tomato lectin-conjugated nanoparticles in the rat. Pharm. Res. 14, 613–618.

Jani, P.U., Halbert, G.W., Langridge, J., Florence, A.T., 1990. Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency. J. Pharm. Pharmacol. 42, 821.

Jani, P.U., Nomura, T., Yamashita, F., Takakura, Y., Florence, A.T., Hashida, M., 1996. Biliary excretion of polystyrene microspheres with covalently linked FITC fluorescence after oral and parenteral administration to male Wistar rats. J. Drug Target. 4, 87–93.

O'Donnell, G.B., Reilly, P., Davidson, G.A., Ellis, A.E., 1996. The uptake of human gamma globulin incorporated into poly(D,L-lactide-co-glycolide) microparticles following oral intubation in Atlantic salmon, *Salmon salar*. Fish Shell. Immun. 6, 507–520.

Pappo, J., Ermak, T.H., 1989. Uptake and translocation of fluorescent latex particles by the rabbit Peyer's patch follicle epithelium: a quantitative model for M-cell uptake. Clin. Exp. Immun. 76, 144–148.

Russell-Jones, G.J., Veitch, H., Arthur, L., 1999. Lectin-mediated transport of nanoparticles across caco-2 and OK cells. Int. J. Pharm. 190, 165–174.

Verzar, F., 1936. Absorption from the Intestine. Longmans, Green and Co, London.

Wells, C.L., Maddaus, M.A., Erlandsen, S.L., 1988. Evidence for the phagocytic transport of intestinal particles in dogs and rats. Infect. Immun. 56, 278–282.