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Research paper

Labelling peptides with fluorescent probes for incorporation into degradable polymers

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Abstract

Two peptides, atrial natriuretic peptide (ANP) and salmon calcitonin (sCT) were conjugated with a fluorescent, amine-reactive probe 5-(and 6-)carboxytetramethylrhodamine,-succinimidylester (5-(6)-TAMRA-SE). The labelling reaction was followed by HPLC and found to be complete after 2 h. The labelled peptides were purified by gel filtration chromatography and characterised by [^{1}H]NMR, UV/VIS and fluorescence spectroscopy. NMR-spectra confirmed the conjugation of dye to the peptides. Two absorption maxima between 500 and 600 nm were recorded in the UV/VIS-spectra. The fluorescence spectra were found to be pH-dependent, which allowed the measurement of pH in aqueous solution. The labelled peptides were encapsulated into poly(lactic acid) (PLA) microspheres using a double emulsion technique. Probe attachment permitted location of the peptides in the polymer. © 1998 Elsevier Science B.V.

Keywords: Peptide labelling; Fluorescent bioconjugate; Fluorescence; pH; Degradable polymer; Microsphere; Poly(lactic acid)

1. Introduction

The therapeutic use of peptides in pharmaceutical formulations faces certain obstacles such as delicate stability or low bioavailability due to enzymatic degradation in body fluids [1]. To improve the ability to deliver peptide and protein drugs, many research groups incorporate these drugs into degradable polymers [2–4]. The resulting micro- or nanoparticulate systems ensure therapeutic plasma levels by protecting peptides from inactivation [5] and by releasing them over a designated period of time [6]. Surprisingly, only a few peptide microsphere preparations have been introduced to the market thus far. One of the reasons could be the interaction of polymer degradation and erosion products with the encapsulated drugs. pH-changes inside the microspheres, for example, could have a destructive effect on the structural integrity of the biomolecules. Another problem can be the adsorption of peptides and proteins at the surface of microparticles instead of encapsulation inside [7], which results in unfavourable release profiles.

Many of these problems could be solved if proteins and peptides could be located more easily after encapsulation. Peptides and proteins have no natural colour and their self-fluorescence in the UV-range depends on the presence of aromatic amino acids (phenylalanine, tyrosine and tryptophane) as well as on the structure of the polypeptide chain [8], but they can be attached to compounds with characteristic properties. Among the most frequently used labelling methods are the coupling of radionucleotides [9] or fluorescent probes [10] to peptides or proteins. Radiolabelling needs special equipment and training, which may not be readily available. Fluorescence labelling, in contrast, is simple and can be performed using standard equipment.

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Conjugates of peptides or proteins with fluorescent dyes have the following advantages:

The labelled drug becomes visible and can be located during the manufacturing of microspheres and in all studies thereafter by fluorescence microscopy [11].

Drug release can be followed more precisely since fluorescent assays have a sensitivity that is three to four orders of magnitude higher than that of standard UV/VIS-methods [12].

Internal changes in the chemical environment inside microspheres during erosion, such as changes in pH, could be detected if the fluorescent label was pH-sensitive.

Although the labelling of proteins and peptides can be achieved with well-established chemical reactions [13], little information on the application of fluorescent labelled peptides and proteins for incorporation into biodegradable polymers is available in the literature. The intention of this work was to use an efficient labelling procedure and to develop suitable purification protocols. The final goal was to characterise the obtained peptide-dye conjugates and to incorporate them into biodegradable microspheres.

2. Materials and methods

2.1. Materials

Atrial natriuretic peptide (α-hANP; lot 3692101) was obtained from Suntory BioPharma Tech Center (Gunma, Japan) and salmon calcitonin (sCT; lot 39060) was from Sandoz (Basel, Switzerland). 5- (and 6-)Carboxy tetramethylrhodamine,-succinimidylester (5-(6)-TAMRA-SE) and its single isomers, were purchased from Molecular Probes (Oregon, USA). Poly(D,L-lactic acid) (PLA, Resomer® 202) from Boehringer Ingelheim (Ingelheim, Germany) was used as a model polymer for encapsulation studies. HPLC grade acetonitrile from Roth (Karlsruhe, Germany) and trifluoroacetic acid (TFA) from Sigma (Deisenhofen, Germany) were used as HPLC solvents. For encapsulation studies 98% hydrolysed poly(vinyl alcohol) (average M_r 13 000–23 000) from Aldrich (Milwaukee, WI, USA) was used as a surfactant. Sephadex G-10 and all other chemicals in analytical grade were purchased from Sigma.

2.2. Peptide labelling with amine-reactive fluorescent dyes

Five milligrams of peptide was dissolved together with 150 mg urea in 0.5 ml 0.1 M sodium bicarbonate solution (pH 8.5). Ten milligrams of 5-(6)-TAMRA-SE in 0.75 ml acetonitrile was added to the peptide solution and stirred with a magnetic stirrer at room temperature. For kinetic studies, samples of 50 μl were taken out of the reaction mixture at regular times and the reaction was quenched by adding 50 μl of 1 M acetic acid.

2.3. HPLC analysis of dye and peptides

Dye, peptides and bioconjugates were analysed by reversed phase HPLC using a 210 × 5 mm Vydac RP-18 column (218TP54) with 5 μm pore size from Sigma. The set-up consisted of an ISS-200 autosampler, a series 200 pump, and a 235 C diode-array detector, controlled by Turbochrom software from Perkin Elmer (Überlingen, Germany). Alternate detection was from a UV/VIS-detector 430 from Kontron (Neufahrn, Germany) and a RF-1501 spectrofluorophotometer from Shimadzu (Kyoto, Japan). The kinetic samples obtained from the labelling reaction were diluted to 1 ml with distilled water. 100 μl were injected and analysed for the different components involved in the reaction. The linear gradient used for ANP was 41–70% phase B in phase A (A: 0.19% TFA in water; B: 0.1% TFA in water/acetonitrile = 1:1) over 25 min. For sCT the linear gradient employed was 80–20% phase A in phase B (A: 0.1% TFA in water; B: 0.1% TFA in acetonitrile) over 25 min. The flow rate was 1.5 ml/min. Peaks were detected at 210 and 255 nm using the diode-array detector, at 550 nm using the UV/VIS-detector and at 576 nm using fluorescence detection after excitation (Ex) at 550 nm. UV/VIS-spectra of detected peaks were recorded in the range of 200 to 360 nm using the diode-array detector.

2.4. Purification of conjugates

Excess dye and other low molecular weight compounds were separated from the labelled peptide by gel filtration chromatography. Sephadex G-10 with a fractionation range above M_r 700 was used as a stationary phase on a 600 × 16 mm i.d. Superformance® column from Merck (Darmstadt, Germany). A mixture of 1 M acetic acid/acetonitrile = 9:1 was used as a mobile phase. The flow rate of 0.4 ml/min was adjusted with a constaMetric 3000 pump from Milton Roy (Florida, USA). The fractions were detected at 550 nm using a UV/VIS-detector 430 from Kontron (Neufahrn, Germany). The conjugate fraction was collected and freeze-dried with a freeze-drier Delta 1–24 KD from Martin Christ (Osterode, Germany).

2.5. Nuclear magnetic resonance (NMR) measurements

Fluorescent dyes and labelled peptides were characterised by [^{1}H]NMR. Spectra were obtained from 1% solutions in DMSO using a BZH 360/52 FT-NMR spectrometer from Bruker (Rheinstetten, Germany) at 360.14 MHz. Tetramethylsilane was used as the internal standard. All δ values are given in ppm.

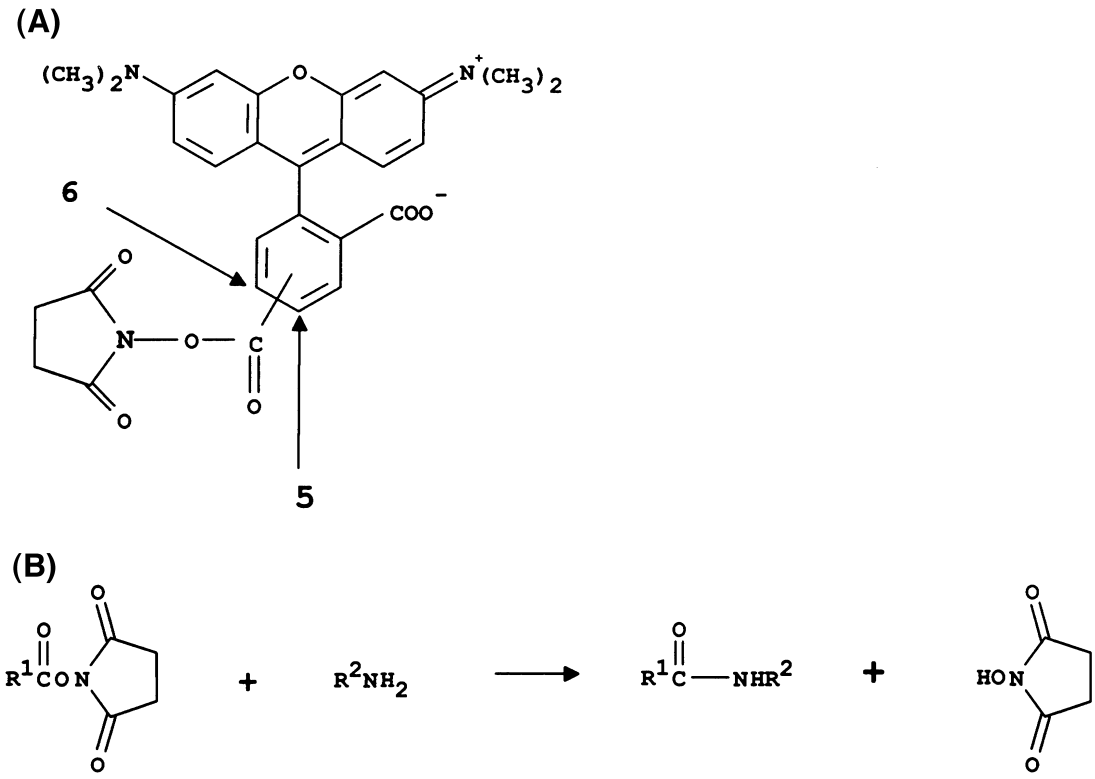


Fig. 1. (A) 5- (and 6-)Carboxytetramethylrhodamine-succinimidylester. (B) Reaction of a primary amine with a succinimidylester (R^1 = TAMRA, R^2 = peptide).

2.6. Characterisation of labelled peptides by UV/VIS and fluorescence spectroscopy

Absorption spectra of 5-(6)-TAMRA-SE, ANP, sCT and dye-peptide conjugates were recorded in the range of 200 to 800 nm with a Uvikon 810 UV/VIS spectrophotometer from Kontron (Neufahrn, Germany). Fluorescence emission (Em) spectra were taken on an RF-1501 spectrofluorophotometer from Shimadzu after excitation at 530 nm. The dye was dissolved in methanol, the peptides and the peptide-dye conjugates in 1 M acetic acid. The pH-dependent spectra of labelled peptides were taken from 0.5 mg/ml and 0.05 mg/ml solutions in citrate-phosphate buffer in the range of pH 2.2 to 8.

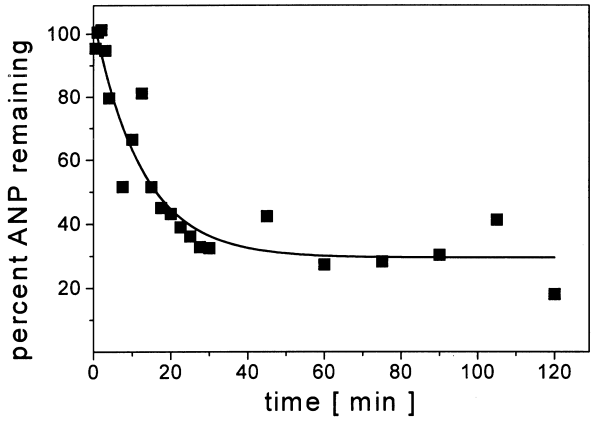


Fig. 2. Reaction kinetics of ANP labelling determined with HPLC at 210 nm.

2.7. Microsphere preparation

PLA microspheres were prepared by solvent evaporation using a double emulsion technique [14]. In brief: labelled peptide was dissolved in 1 M acetic acid to give a concentration of 0.5 mg/ml. 200 μl of the peptide solution were dispersed in a solution of 300 mg Resomer® 202 in 1 ml methylenchloride. This water/oil (w/o) emulsion was homogenised for 90 s with a Branson Sonifier B-12 (Branson,

Table 1

Components of reaction mixture determined with HPLC

	ANP	Labelled ANP	Reactive dye	Unreactive dye
Retention time (min)	13.23	14.36	23.30; 27.24	17.63–20.53
	sCT	Labelled sCT	Reactive dye	Unreactive dye
Retention time (min)	19.65	21.66–23.45	14.50; 16.26	9.29–12.86

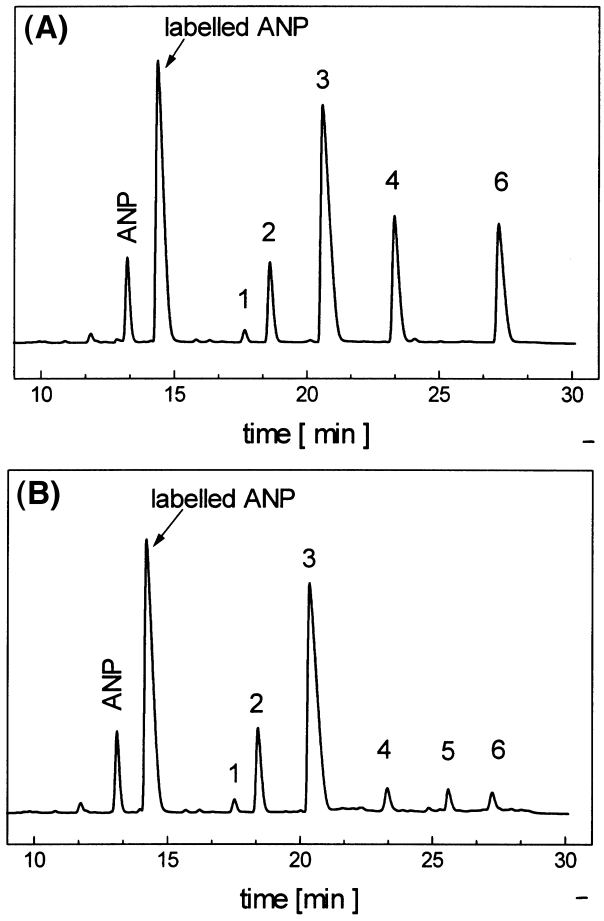


Fig. 3. (A) HPLC chromatogram of labelling mixture after 0.5 min reaction time (255 nm). Peaks 1 to 6: fluorescent dye; peaks 4 and 6: reactive components. (B) HPLC chromatogram of labelling mixture after 20 min reaction time (255 nm). Peaks 1 to 6: fluorescent dye; peaks 4 and 6: reactive components; peak 5: hydrolysation product.

Carouge-Genève, Switzerland). After adding of 2 ml 0.3% aqueous poly(vinyl alcohol) solution under vortex mixing a multiple emulsion formed spontaneously and was poured into a 0.1% poly(vinyl alcohol) solution. The organic solvent was evaporated for 2 h. The resulting microspheres were collected by centrifugation at 1500 rpm (Centrikon T-42 K, Kontron), washed with distilled water and freeze dried. Photographs were taken during all preparation steps with a fluorescence microscope. The microscope set-up comprised a BH2-RFCA microscope, a BH2-RFL-T3 burner and a SC35 Type 12 camera from Olympus Optical Co. (Tokyo, Japan), and an HBO 100W/2 mercury short arc lamp from Osram (Berlin-München, Germany).

Table 2

Components of 5- (and 6-)TAMRA-SE in acetonitrile/water determined with HPLC (Ex 530 nm, Em 576 nm)

Peak no.	Retention time (min)	Percent of total amount injected	Assignment	Reactive
1	9.19	4.5	5-isomer	No
2	10.22	12.5	6-isomer	No
3	11.69	4.1	5-isomer	No
4	12.95	15.2	5-isomer	No
5	14.49	24.6	6-isomer	Yes
6	16.26	39.1	5-isomer	Yes

HPLC conditions were the same as for sCT.

3. Results and discussion

The conjugation of amines with activated esters is an established method to form new *amide* bonds. In the same way tetramethylrhodamine, a fluorescent dye, can be attached to proteins and peptides, if it has been activated

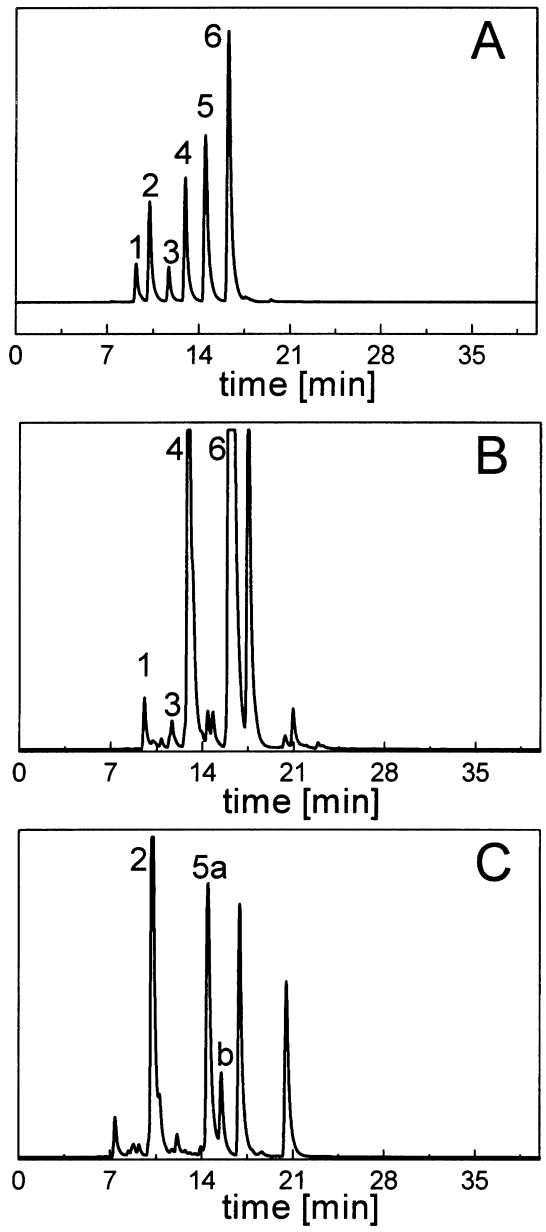


Fig. 4. (A) HPLC chromatogram of 5-(6)-TAMRA-SE (Ex 530 nm, Em 576 nm). (B) HPLC chromatogram of 5-TAMRA-SE (Ex 530 nm, Em 576 nm). (c) HPLC chromatogram of 6-TAMRA-SE (Ex 530 nm, Em 576 nm).

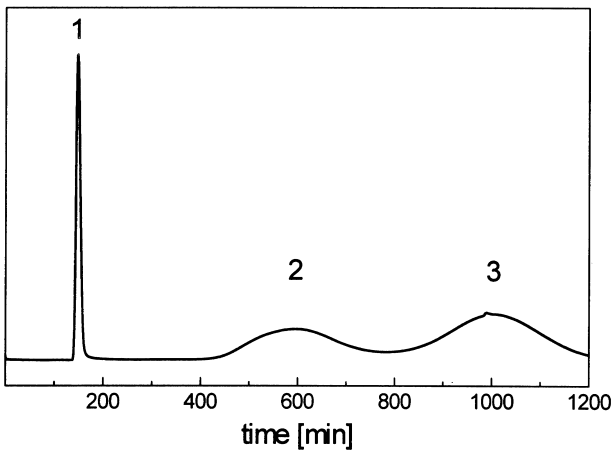


Fig. 5. Gel-filtration chromatogram of labelled ANP. 1, labelled ANP; 2, unhydrolysed dye; 3, hydrolysed dye.

by an acylating substituent, e.g a succinimidylester (Fig. 1A). By reacting with primary aliphatic amines such as from the terminal amino group or side chain amino acids, the dye then is covalently bound to the peptide (Fig. 1B). Succinimidyl esters are ideal reagents for the activation of dyes, since they require only a mildly basic pH of 8.5 to react with the unprotonated amines and to form stable amide bonds [15]. The reaction mechanism is well established [16] and consists of the nucleophilic amine component attacking the carboxylic C-atom of 5-(6)-TAMRA-SE by forming a hydrogen-bonded transition state, wherein the amine component is fixed to a favourable reaction position. The subsequent rapid cleavage of water soluble *N*-hydroxysuccinimide is related to its electron-attracting effect and the peptide-dye product is left. Special attention had to be paid to the low solubility of the peptides [17,18], since the labelling reaction required slightly basic media close to their isoelectric point. An intermediate pH between the optimal pH for the labelling reaction and the pH for peptide solubility had to be found. To improve the solubility of the two model peptides, urea was used as a solubilizer. Concentrated solutions of urea are solvents, in which polypeptide chains are as close to the unperturbed state as possible and therefore mostly soluble [19].

To determine the end point of the reaction, the kinetics were investigated. The peptide disappeared from the reaction mixture in an exponential way (Fig. 2). More than 50% of peptide was labelled after 20 min, and after 1 h the reaction was complete. The retention times of the peaks in the HPLC chromatograms obtained from the reaction mixtures of both peptides are shown in Table 1.

The chromatogram of ANP was found to contain seven different components immediately after the reaction was initiated (Fig. 3A). After 20 min the number of components increased to eight (Fig. 3B). Two peaks represent ANP and its bioconjugate, the other six stem from the fluorescent dye. Only two of the dye peaks decreased during the reaction, therefore one can conclude that these represent the two reactive isomers of 5-(6)-TAMRA-SE. The other peaks represent unreactive components. In addition 5-(6)-TAMRA-SE may contain the free, unreactive dye. To clarify this, 5-(6)-TAMRA-SE was investigated by HPLC. In fact both UV/VIS detection and fluorescence detection (Fig. 4A–C) of the dye in an acetonitrile/water solution revealed more than the expected number of dye compounds. Since activated esters with acylating groups such as succinimidyl esters are prone to degradation in water [15], the additional peaks were suspected to be hydrolysis products. To identify the peaks, 5-TAMRA-SE and 6-TAMRA-SE were also investigated by HPLC. By comparison with fluorescence chromatograms of the single isomers (Fig. 4B and C), the different peaks of 5-(6)-TAMRA-SE were assigned to either the 5- or 6-isomer. Each isomer contributes two major compounds; components 1 and 3 appeared to be impurities of 5-TAMRA. The fluorescence chromatograms showed a large amount of small peaks, which are probably impurities. This is in agreement with findings by other authors, who have described a high heterogeneity of tetramethylrhodamine derivatives [20]. Considering these facts, the two reactive compounds belonging to the succinimidyl-isomers could be identified beyond a reasonable doubt, but it appears impossible to identify all the peaks or to distinguish between impurities and possible hydrolysis products. Table 2 gives an overview of HPLC data for 5-(6)-TAMRA-SE.

Excess dye and low-weight impurities were removed from the bioconjugate by gel filtration chromatography. The reaction mixture yields three fractions, the first distinct one being the dye-peptide conjugate. Labelled ANP could be collected after 2.5 h (Fig. 5) from the exclusion volume, labelled sCT after 1.5 h, respectively (Fig. 6). They were followed by two rather broad peaks. These peaks represent unconjugated, unhydrolysed (peak 2) and unconjugated, hydrolysed dye (peak 3). The amount of hydrolysed and unhydrolysed dye in the reaction mixture of ANP were roughly the same. The amount of hydrolysed dye in the sCT reaction mixture is approximately 3-times larger than the unhydrolysed one.

NMR-spectroscopy confirmed the conjugation of dye to

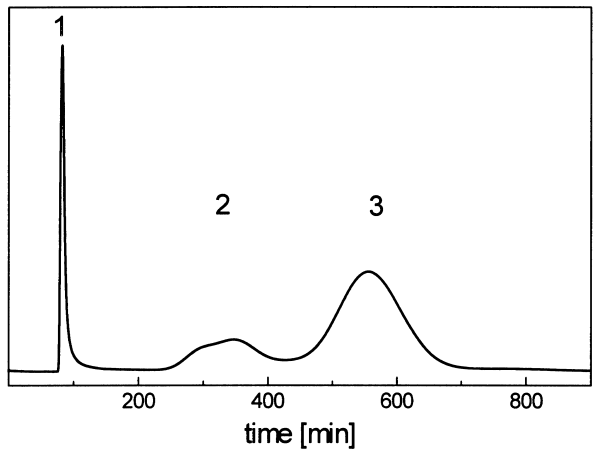


Fig. 6. Gel-filtration chromatogram of labelled sCT. 1, labelled sCT; 2, unhydrolysed dye; 3, hydrolysed dye.

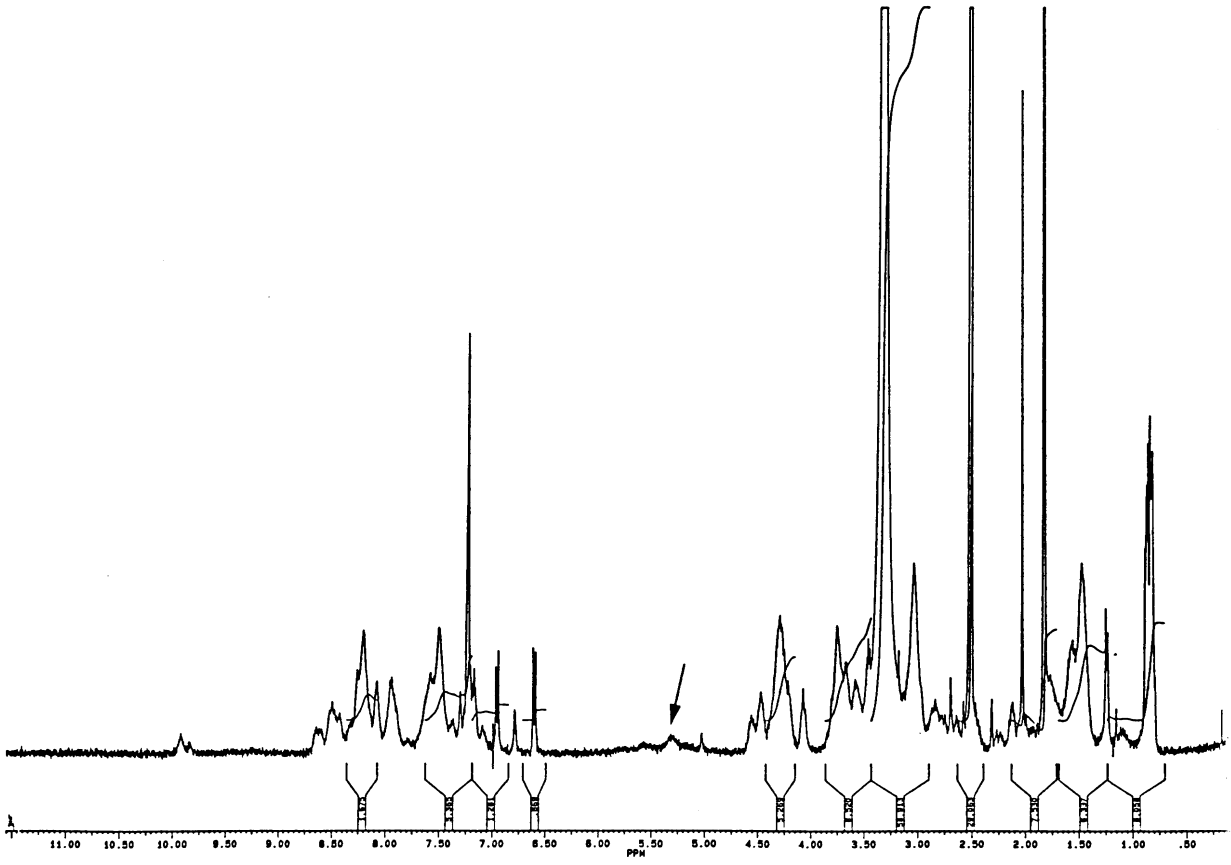


Fig. 7. [^{1}H]NMR spectra of ANP (arrow indicates the signal from the primary amino groups).

the peptides (Figs. 7 and 8). The signal at 5.33 ppm, which may be assigned to the primary amino group, disappeared after the labelling reaction, which indicates that the dye is attached to the N-terminal amino group. It cannot, however, be excluded that dye molecules are also attached elsewhere [10]. At pH 8.5 phenolic hydroxyl groups such as the 4-hydroxy group of tyrosine, for example, are unprotonated and could react with 5-(6)-TAMRA-SE as well. To investigate this possibility, dissociable protons were removed from the peptides by adding deuterated water. Thus, only the undissociable protons of the aromatic compounds remained. The NMR spectra of ANP displayed two distinct signals after this proton exchange, which resulted from the amino acids tyrosine and phenylalanine. After labelling with 5-(6)-TAMRA-SE the signal splitting of tyrosine disappeared and the coupling constant was different, which indicates that at least a part of the tyrosine groups in the ANP molecule were modified.

To further prove that the labelling reaction was successful, absorption spectra were recorded. At first, dye and peptides were investigated. Both peptides showed maximal absorption between 200 and 220 nm. In contrast to the spectra of the unconjugated peptides, two peaks in the visible range appeared at 522 and 558 nm for labelled sCT and at 523 and 558 nm for labelled ANP (Fig. 9). The peak splitting, which is different from the value that was found for the free dye in methanol, is a typical phenomenon for tetramethylrhodamine labelled proteins [21]. It arises from the varying degree of substitution of dye per biomolecule and complicates the determination of dye/peptide level.

One of the major goals for the manufacture of peptide-dye conjugates was to facilitate the location of the peptides inside microspheres after encapsulation. 5-(6)-TAMRA-SE was chosen as fluorescent label, because it shows less photobleaching than other fluorescent derivatives such as fluorescein, for example [22]. Rapid fading of the dye would complicate fluorescence microscopy. Furthermore, photodegradation is not desirable for quantitative measurements during release studies. Although 5-(6)-TAMRA-SE fluorescence is pH-insensitive according to the literature [20,21], the fluorescence of peptide-dye conjugates was critically examined for its spectral properties in citrate-phosphate buffer solutions of pH 2.2 to 8 (Fig. 10). A clear pH-dependence of fluorescence intensity was observed, which was not shown in the corresponding absorption spectra. It can be concluded that in contrast to the parent dye, the resulting bioconjugates are sensitive to variations of pH. This phenomenon could be related to changes in dye properties after the reaction or to conformational changes of the peptide

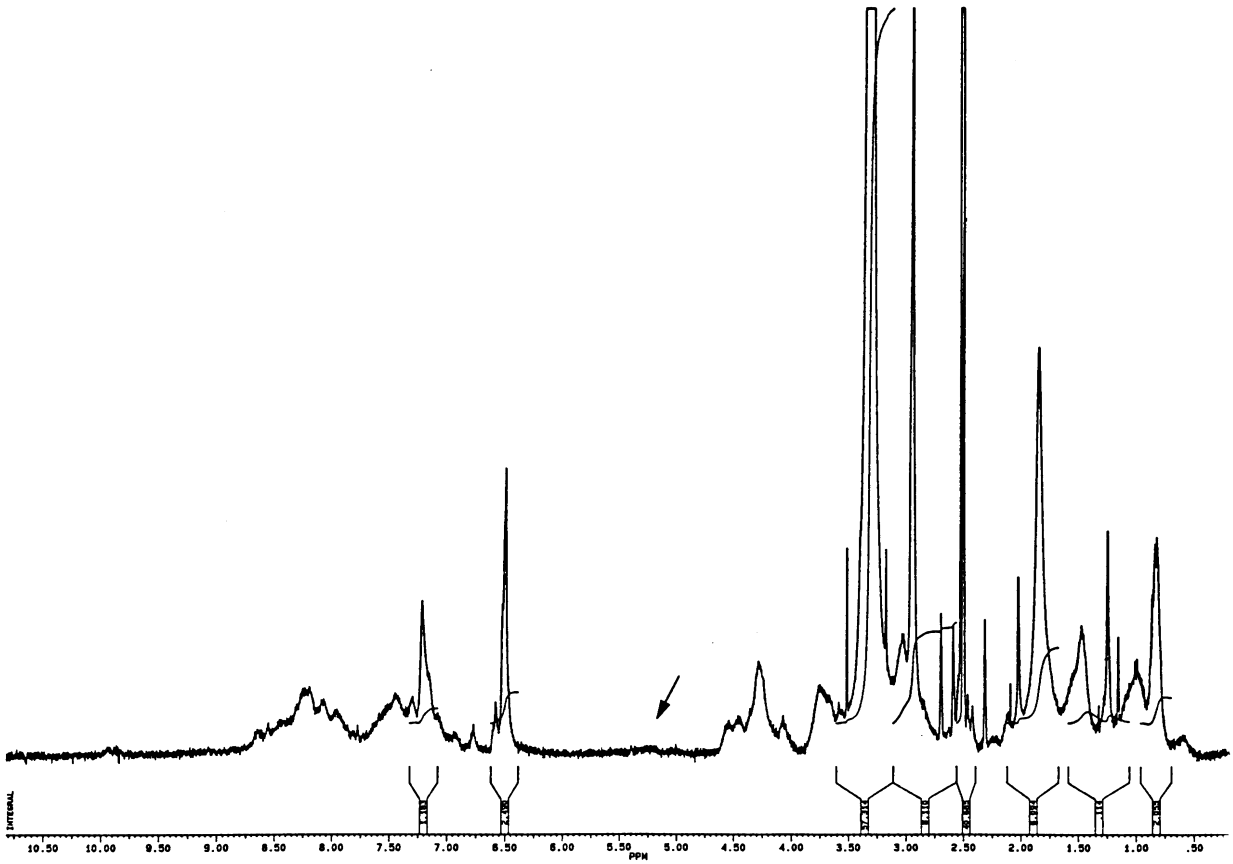


Fig. 8. [^{1}H]NMR spectra of labelled ANP (arrow indicates that the signal from primary amino groups has disappeared).

backbone. At physiological pH-values the p*K* of 5-(6)-TAMRA-SE's tertiary amino group is too high for an ionisation change, but progressive fluorescence quenching with decreasing pH has been reported [23]. On the other hand, structural fluctuations of the peptide induced by environmental conditions, such as pH, may also be involved [24]. The pH-dependent fluorescence of the conjugates could be of great value for studying pH-changes inside eroding microspheres made of biodegradable polymers. Changes could be detected directly at the peptide, as the label is covalently fixed to the drug. This would be a major advantage over other approaches, in which the pH-probe is independent from the peptide and can move away freely. However, many problems have still to be solved prior to the use of the conjugates as pH-sensitive probes. The system has to be calibrated, and a measuring technique has to be used that allows to eliminate the impact of light-scattering on the results.

In order to follow the fate of the drug throughout all preparation steps, the labelled peptides were encapsulated

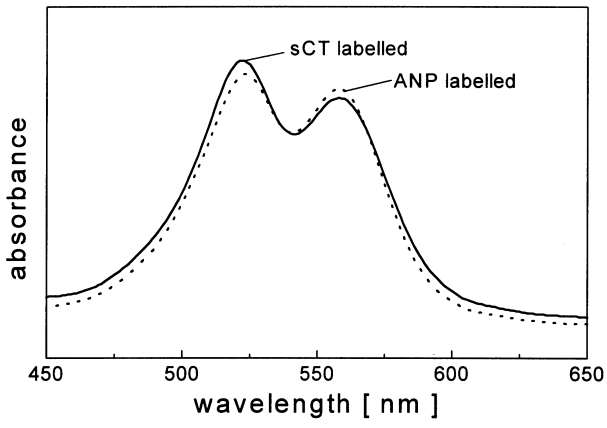


Fig. 9. Absorption spectra of labelled ANP and sCT.

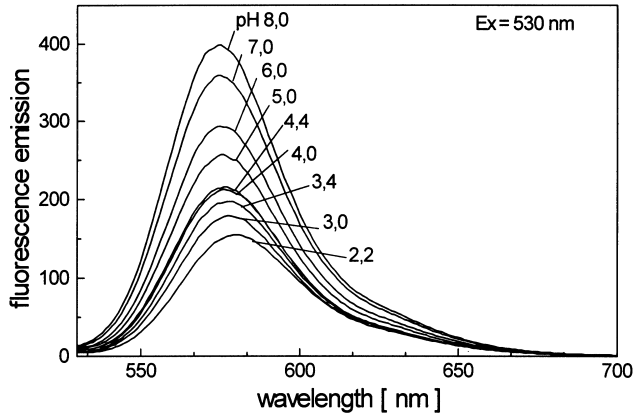
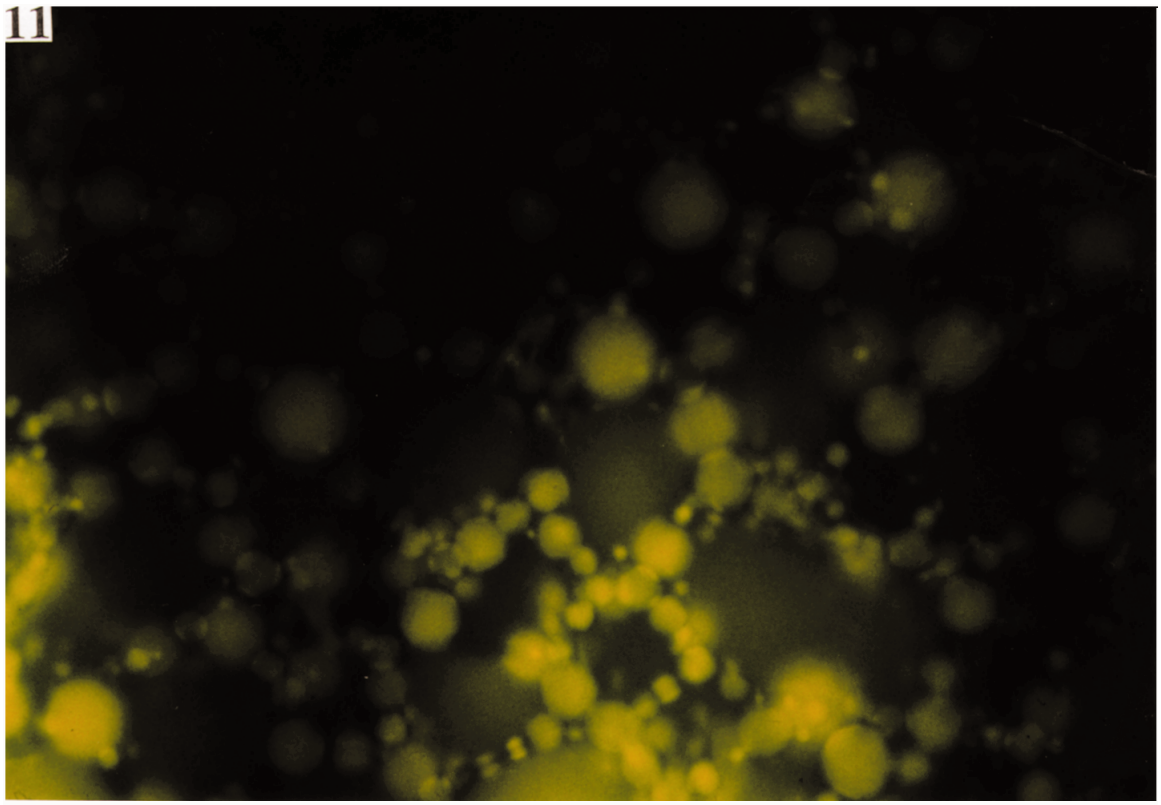


Fig. 10. Fluorescence emission spectra of labelled ANP in citrate-phosphate buffer of different pH.



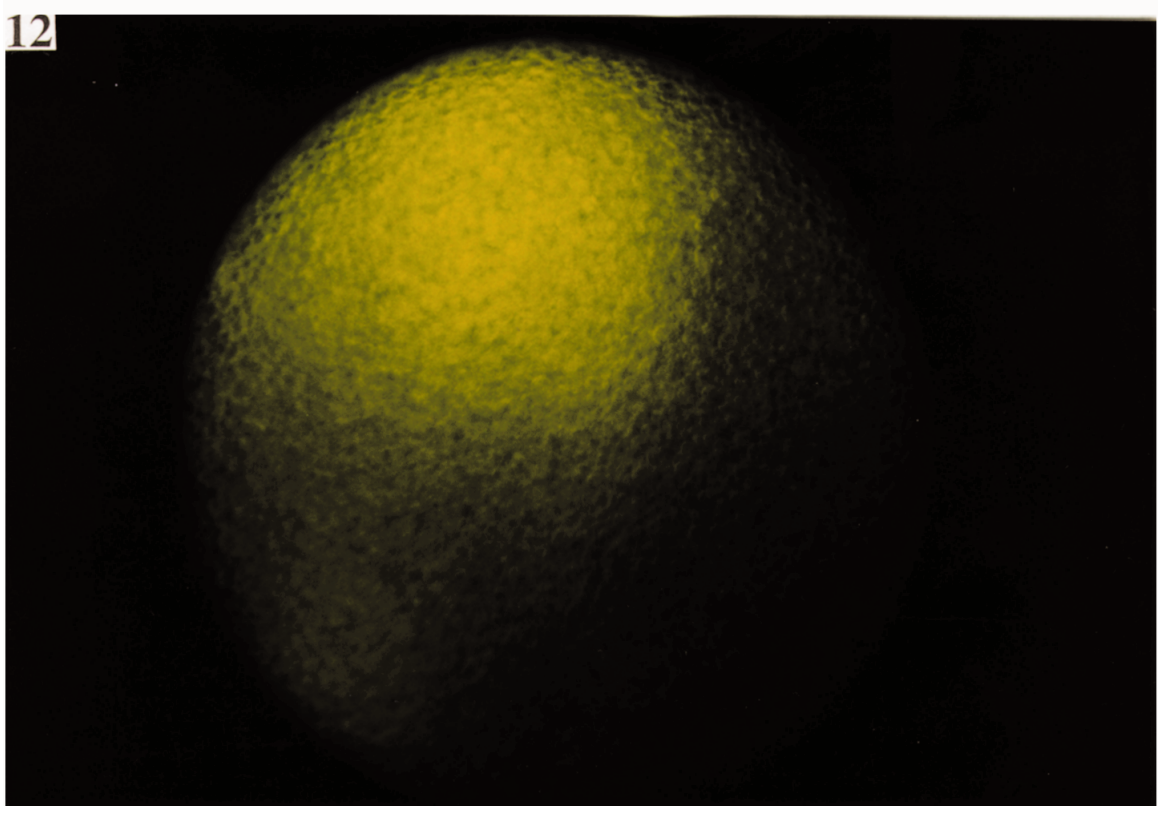


Fig. 11. Picture of the w/o-emulsion containing labelled ANP taken with a fluorescence microscope.

Fig. 12. Picture of microspheres containing labelled ANP taken with a fluorescence microscope.

into PLA microspheres and located by fluorescence microscopy. Peptide sites in the w/o-emulsion of aqueous peptide and organic polymer solution (Fig. 11) could be seen clearly. The drug gathered in the fast coalescing water droplets, which were dispersed in the polymer matrix. After encapsulation the labelled peptides were distributed all over the microspheres in a pattern similar to the one observed in the w/o-emulsion (Fig. 12). It became evident that particles had a blister-like surface, as long as the polymer matrix was not completely hardened. Afterwards fluorescence became more bright and the microsphere shape could be seen distinctly. The labelled compounds proved to be excellent materials to investigate the fate of peptides during microencapsulation.

4. Conclusions

The proposed method for labelling peptides with fluorescent dyes and the subsequent purification by gel filtration proved to be simple, quick and efficient. Bioconjugates could be identified by their characteristic absorption and fluorescence spectra. The fluorescence emission of peptide-dye conjugates proved to be pH-dependent. With the help of labelled peptides, drug sites throughout formulation and in the resulting drug delivery systems could be detected with great specificity.

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