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STRATEGIES FOR LABELLING BRANCHED POLYPEPTIDES WITH A POLY(L-LYSINE) BACKBONE WITH RADIOIODINES (^{123}I, ^{125}I, ^{131}I) AND RADIOMETALS (^{111}In, ^{51}Cr) FOR BIODISTRIBUTION STUDIES AND RADIOPHARMACEUTICAL DEVELOPMENT

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SUMMARY

Methods have been developed for radiolabelling synthetic branched polypeptides, these being based on a poly(L-lysine) backbone with short side chains of three DL-alanine residues and one other amino acid at the end of the branches (MW ~45-100 kDa). Labelling has been carried out with gamma emitting radionuclides suitable for use in biodistribution studies or for gamma scintigraphy. Labelling with ^{125}I was achieved by reaction of the polypeptides' terminal amino groups with pre-iodinated Bolton and Hunter Reagent (*N*-Succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate). Alternatively, polypeptides were reacted with non-labelled Bolton and Hunter reagent, which could subsequently be iodinated with ^{123}I, ^{125}I or ^{131}I by oxidative incorporation from [^{123}I]NaI, [^{125}I]NaI or [^{131}I]NaI. For labelling with radiometals, the polypeptides' terminal amino groups were reacted with diethylenetriaminepentaacetic acid (DTPA) anhydride, and the conjugated DTPA subsequently labelled with ^{111}In or ^{51}Cr by chelation. An amphoteric polypeptide, having terminal glutamic acid residues on its side chains (EAK), and a polycationic polypeptide, with terminal D-lysine (D-KAK) were labelled in these ways. In addition EAK previously conjugated to the *cis*-aconityl derivative of daunomycin (EAK-cAD) was similarly labelled. Gel permeation chromatography on Sephacryl S-300, which was possible with the amphoteric EAK, showed virtually identical elution profiles with ^{123}I, ^{125}I, ^{131}I, ^{111}In and ^{51}Cr labelled EAK and its cAD conjugate. Biodistribution studies in mice showed prolonged blood survival of the radionuclide of ^{125}I, ^{111}In or ^{51}Cr labelled EAK and EAK-cAD. There were, however, differences in organ levels of the radionuclides. Generally kidney, spleen and liver levels of radiometals were higher than those of radioiodine, while levels in the gastrointestinal tract were higher with radioiodine. D-KAK labelled with any of the three radionuclides was cleared rapidly from the blood, high levels of all tracers being found in spleen, liver, kidney and lung.

Keywords Branched polypeptides; Poly-(L-lysine); Radioiodine; ^{111}In, ^{51}Cr, Biodistribution

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INTRODUCTION

Synthetic macromolecules have great potential in a number of areas of clinical application. These include their use as carriers of drugs (for example for cancer chemotherapy [1-4]), antigenic determinants (for vaccines [5,6]), hormones [7,8], and gamma emitting radionuclides (for use as radiopharmaceuticals [9-11]). We have previously described new groups of branched polypeptides potentially suitable for many of these purposes, having the general formula poly[Lys-(X_i-DL-Ala_m)] (XAK), where $i<1$ and $m\tilde{}3$ [12-17]. These polypeptides are water soluble and biodegradable. Their large number of α-amino groups means that they are ideal for simple and efficient conjugation to drugs or other agents. Whatever their clinical use, biodistribution of these branched polypeptides will be all important. We have previously studied the biodistribution of a range of these branched polypeptides, in mice, by radiolabelling them with ^{125}I by reaction of their terminal amino groups with pre-iodinated Bolton and Hunter Reagent (*N*-Succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)-propionate) [17]. Those biodistribution studies showed that branched polypeptides with glutamic acid at the side chain terminal position (EAK), which will be amphoteric under physiological conditions, survive for long periods in the blood. Polycationic polypeptides (regardless of their branch structure) were cleared rapidly from the circulation.

Our later studies showed that it was also possible to label EAK with the radiometal ^{111}In, by chelation to diethylenetriaminepentaacetic acid (DTPA) conjugated to the polypeptide's terminal amino groups by reaction with its bicyclic anhydride [18,19]. ^{111}In has advantages over ^{125}I for biodistribution studies. The major advantage is that its energies of gamma emission (173 keV and 247 keV) mean that it can be efficiently detected by gamma cameras, allowing non-invasive visualisation of its biodistribution, which is essential for clinical biodistribution studies. An additional advantage is that radiometals such as ^{111}In tend to be retained at the site of clearance and catabolism of their carrier, unlike radiohalogens which are cleared rapidly and undergo subsequent urinary excretion. Thus levels of radiometals in tissues give a better picture of the total uptake of their carriers, rather than a level only at the time of analysis. For labelling another class of synthetic macromolecule (a copolymer of styrene and maleic acid, designated

SMANCS) as well as the XAK branched polypeptides, Maeda et al [3,20,21] used ^{51}Cr, also by chelation to DTPA, to take advantage of its residualising properties and to thus to measure the tumoritropic accumulation of polymers in animal tumours.

The objective of the present study was to develop further the strategies for labelling the XAK branched polypeptides with a poly(L-lysine) backbone. Particularly we sought to know: a) Whether reaction with unlabelled Bolton and Hunter reagent could yield products suitable for subsequent iodination with ^{123}I and ^{131}I, since these are suitable for gamma scintigraphy. b) Whether conjugates of the polypeptides with unlabelled Bolton and Hunter reagent or DTPA were stable on prolonged storage, so that they could be labelled with radionuclide when required. c) Whether DTPA derivatives of polypeptide or drug-polypeptide conjugates could be labelled with ^{51}Cr as an alternative to ^{111}In. d) Whether the in vitro characteristics of any one polypeptide were the same, irrespective of the radiolabel used. e) Whether there is any difference in the biodistribution of the various radiolabels from labelled polypeptide, both in the pattern of survival in the blood and in subsequent distribution in various organs. For these studies we have used both amphoteric polypeptide (EAK), expected to show good survival of its radiolabel in the blood, and a polycationic polypeptide (D-KAK), expected to be cleared rapidly.

MATERIALS AND METHODS

Branched polypeptides and their cAD conjugates

Abbreviations used in this paper follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature [22] in accord with the recommended nomenclature of graft polymers [23].

All branched polypeptides were synthesised in our laboratories, using previously described methods. Their primary structure was determined by amino acid analysis, by identification of the branch-terminating residues, and by enantiomer composition of the side chains and size was analyzed by sedimentation analysis and gel chromatography [13-16].

Conjugates between the *cis*-aconityl derivative of daunomycin and branched polypeptides were made by carbodiimide conjugation and their cAD content determined spectrophotometrically as previously described [4].

Table 1 summarises the characteristics of the polypeptides and their cAD conjugates used in the present study.

Table 1 Characteristics of branched chain polypeptides

No.	Abbreviation	Structure	Molar ratio cAD:peptide	M_w^* (±5%)
1	EAK	Poly[Lys-($Glu_{0.88}$-DL-$Ala_{3.19}$)]	-	55,400
2	EAK	Poly[Lys-($Glu_{0.93}$-DL-$Ala_{2.94}$)]	-	45,800
3	EAK-cAD	Poly[Lys-(cAD_j-$Glu_{0.93}$-DL-$Ala_{2.94}$)]	12.5:1	53,800
4	EAK	Poly[Lys-($Glu_{0.79}$-DL-$Ala_{3.35}$)]	-	56,100
5	D-KAK	Poly[Lys-($Lys_{0.87}$-DL-$Ala_{2.95}$)]	-	91,600

* Calculated from the average degree of polymerisation and from the side chain composition.

Labelling of polypeptides with ^{125}I-Bolton and Hunter reagent

Polypeptide #1 (EAK, Table 1) was labelled directly with pre-iodinated Bolton and Hunter Reagent (*N*-Succinimidyl 3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate) [17]. Twenty μL of the reagent (Amersham International, plc, Amersham, UK) was added to a plastic microfuge tube, and evaporated to dryness under a stream of nitrogen. One hundred μg of polypeptide in 100 μL of phosphate buffered saline (PBS) at pH 8.0 was added, and the solution agitated periodically over a 10 minute incubation at room temperature. Subsequently the ^{125}I labelled conjugate was purified by passage through Sephadex G-25, using pre-packed PD-10 columns (Pharmacia, Milton Keynes, UK), elution being in PBS at pH 7.2. Labelling efficiency was 50%, and specific activity was 25 MBq/mg.

Reaction of polypeptides with Bolton and Hunter reagent and labelling with ^{123}I, ^{125}I, and ^{131}I

Polypeptide #2 and its cAD conjugate, polypeptide #3, (Table 1) were conjugated to non-labelled Bolton and Hunter reagent. Coupling was performed with *N*-Succinimidyl 3-(4-hydroxyphenyl)propionate (Sigma Chemical Co. Dorset, UK) in 0.05 M carbonate buffer at pH

9.4. Briefly, dried Bolton and Hunter reagent was dissolved in dimethylsulphoxide (DMSO) at 4.0 mg/mL. From this solution 80 μL was added dropwise to 3 mg of EAK polypeptide or 4 mg of EAK-cAD conjugate dissolved in 0.05 M carbonate buffer at pH 9.4. The mixture was stirred for 30 minutes at room temperature and then dialysed against PBS (pH 7.2). The average degree of molar substitution, determined spectrophotometrically at 274 nm was 3.8 for EAK and 3.5 for EAK-cAD. Solutions were stored at 4^0 C, in PBS at pH 7.2.

Labelling of Bolton and Hunter derivatised EAK and EAK-cAD was carried out with [^{123}I]NaI (^{123}I Iodide Solution for Labelling (B) in 0.01 M NaOH, Medgenix Diagnostics, Milton Keynes, UK), [^{125}I]NaI (^{125}I for Protein Iodination (IMS.30) in NaOH pH 7-11, Amersham International plc, Amersham, UK), or [^{131}I]NaI (^{131}I for protein Iodination (IBS.3) in NaOH pH 7-11, Amersham International), by oxidative incorporation into the phenyl group using Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) as the oxidizing agent. One hundred μg of Iodogen (Pierce and Warriner (UK) Ltd, Chester, UK) was coated onto the inner surface of polypropylene conical microfuge tubes by evaporation under nitrogen from solution in methylene chloride. Two hundred μg of EAK or EAK-cAD in 100-200 μL of PBS (pH 7.2) and 3-15 MBq of radioiodine solutions, were added to the tubes, and the solution mixed with a Pasteur pipette. After incubation at room temperature for 10 minutes, the reaction was stopped by removal of the mixture from the reaction tube. The labelled polypeptides were purified by passage through Sephadex G-25, elution being in PBS at pH 7.2. Specific activities of the final products were 10-30 MBq/mg.

Reaction of polypeptides with DTPA anhydride and labelling with ^{111}In and ^{51}Cr

Polypeptides were conjugated to DTPA by reaction with DTPA anhydride (DTPAA) as described previously [18]. Briefly, to solutions of polypeptides or their cAD conjugates in 0.05 M carbonate buffer at pH 9.4 was added dropwise a solution of DTPAA (Sigma Chemical Co, Dorset, UK) in anhydrous DMSO to give a 3:1 DTPAA:polypeptide molar ratio. After 10 minutes at room temperature, unreacted DTPA and DMSO were removed and the polypeptide-DTPA conjugates transferred by dialysis to either 0.3 M sodium citrate/citric acid buffer (pH 6.0)

or 0.1 M Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid) buffer (pH 7.0). Solutions were stored at 4^0 C.

Labelling with ^{111}In was carried out as previously described [18,19]. Five to ten MBq of ^{111}In[$InCl_3$] (Indium chloride in 0.04 M HCl, INS.1P, Amersham International) were added to solutions of 200-300 μg of polypeptide-DTPA conjugates in 200-300 μL of pH 6.0 citrate buffer. After 10 minutes at room temperature, the reaction mixtures were purified of unbound ^{111}In by passage through Sephadex G-25, elution being in PBS at pH 7.2. Specific activities of the final products were 5-30 MBq/mg.

Labelling with ^{51}Cr was based on the method of Maeda [3,20,21]. Ten to fifteen MBq of ^{51}Cr[$CrCl_3$] (Chromium(III) chloride in 0.1 M HCl, CJS.2, Amersham International) were added to 300-500 μg of polypeptide-DTPA conjugates in 200-300 μL of pH 7.0 Hepes buffer. The reaction mixture was left at 4^0 C overnight, and subsequently purified of unbound ^{51}Cr using Sephadex G-25, with elution in PBS at pH 7.2. Specific activities of the products were 10-20 MBq/mg.

Radionuclide counting

During labelling procedures, radioactivity measurements were made with an Isocal radionuclide calibrator (Vinten Instruments, Surrey UK). Radionuclides were counted in in vitro and in vivo samples with an LKB 1282 Compugamma CS (Pharmacia LKB, Milton Keynes, UK). In some cases ^{125}I and ^{111}In were counted simultaneously, using the Compugamma's Multi-isotope option.

Gel permeation chromatography

Gel permeation chromatography of radiolabelled polypeptides was carried out on columns of Sephacryl S-300 Superfine (Pharmacia) [19]. Column dimensions were 1.8 x 90 cm, elution was in PBS at pH 7.2 at a flow rate of 20 mL/hour, with automatic collection of fractions of 1.5 or 2.0 mL. The void volumes were determined using Blue Dextran (Pharmacia).

Blood clearance and biodistribution studies

All in vivo studies were carried out in female Balb/c mice (Biomedical Services Unit, University

of Nottingham) with appropriate Licences from the UK Home Office, and with due consideration for animal welfare.

Groups of mice (n=4) were injected intravenously via a tail vein with 5-10 μg of labelled polypeptides in 0.2 mL of PBS (pH 7.2). In some cases an ^{125}I labelled preparation was injected admixed with an ^{111}In labelled. Serial blood samples (10 μL) were taken from the tail tip at 1, 10 and 30 minutes and 2 and 3 hours after injection directly into microcapillary pipettes (Drummond Microcaps, Drummond Scientific Co., Broomhall, PA, USA).

At four hours after injection, mice were killed, and weighed samples of blood, visceral organs and residual carcass assayed for radioactivity.

To construct time:activity blood clearance curves, count rates in the total intra-vascular blood volumes were calculated assuming the blood volume of the mice (in mL) to be 11.2% of the body weight (in g) [17] and expressed as a percent of the total initially injected count rate. Areas under curves, as percent dose:hours, were calculated using the trapezoidal rule [17]. Results of the tissue distribution analysis are expressed as a percentage of the total initially injected count rate recovered per g of tissue.

RESULTS AND DISCUSSION

Effect of storage on DTPA and Bolton and Hunter derivatised polypeptides

Three of the current preparations were stored for over two years in solution at 4^0 C, with no detrimental effect on radionuclide labelling efficiency or in vitro characteristics. Thus with polypeptide #2 (Table 1) its DTPA conjugate was stored in citrate buffer pH 6.0, suitable for ^{111}In labelling by direct addition of $^{111}InCl_3$, for 2¼ years, and still labelled with virtually 100% efficiency, and gave the same gel filtration elution profile on S-300 (see below). Similarly its Bolton and Hunter conjugate, and the Bolton and Hunter conjugate of its cAD conjugate (polypeptide #3), stored in PBS at pH 7.2, labelled with virtually 100% efficiency after two years' storage, and still gave the same gel permeation elution profile.

Gel permeation chromatography

Gel permeation chromatography showed all of the labelled EAK and cAD-EAK preparations

eluting in a broad peak, starting shortly after the void volumes of the columns, with small shoulders of later eluting material (Figures 1-3). These elution profiles were essentially the same as those previously reported with ^{111}In labelled EAK [18,19]. This broad elution profile is probably the result of heterogeneity of conformation of these molecules, which is known from circular dichroism studies to be relatively unordered [14]. (The presence of the later eluting shoulder is indicative of a lower molecular mass component in the preparations, data not shown.) The important observation in the present study was that the elution profile of any one preparation was virtually identical, irrespective of how it had been labelled or whether it had also been conjugated to cAD. Thus Figure 1 shows very similar patterns with polypeptide #2 (Table 1) when it had been labelled with ^{111}In following reaction with DTPAA or with ^{123}I or ^{131}I following reaction with Bolton and Hunter Reagent. Figure 2 shows the profiles of the same preparation of polypeptide labelled again with ^{111}In, or with ^{125}I, or its cAD conjugate (polypeptide #3, Table 1) labelled with ^{111}In, or with ^{125}I. Figure 3 shows elution of polypeptide #4 (Table 1) labelled

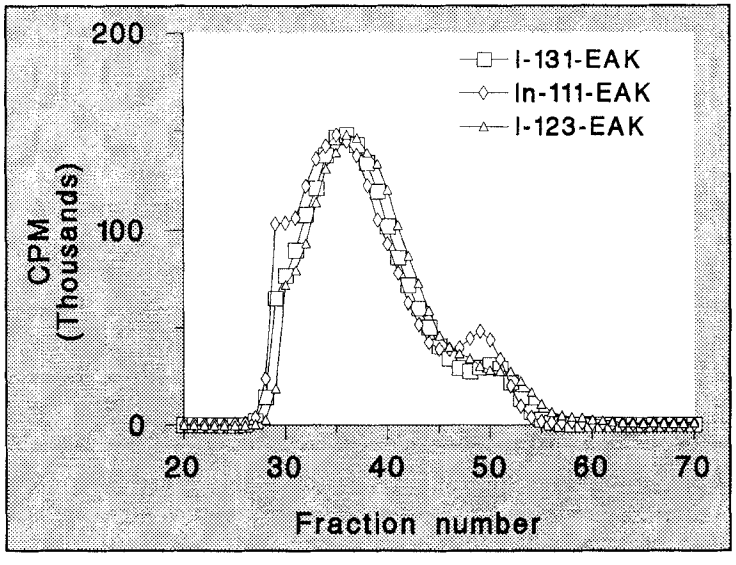

Figure 1 S-300 elution profiles of EAK (polypeptide #2) labelled with three different radionuclides. EAK was conjugated to Bolton and Hunter Reagent for subsequent labelling with ^{123}I or ^{131}I, or DTPA for ^{111}In labelling. The void volume of this column was such that Blue Dextran eluted at fraction 28. Count rates have been normalised to give the same heights for the major peaks.

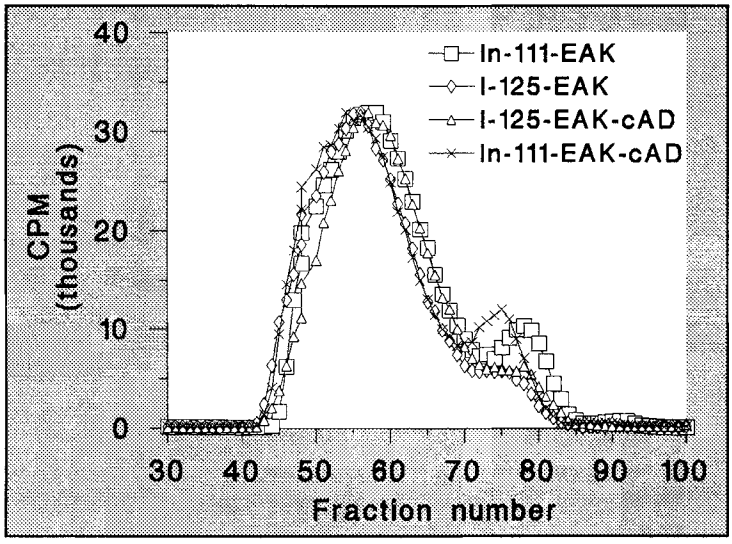

Figure 2 S-300 elution profiles of EAK, or its cAD conjugate (polypeptides #2 and #3) labelled with ^{111}In by DTPA chelation or ^{125}I following reaction with Bolton and Hunter Reagent. This is a different S-300 column from that in Figure 1, but the same as in Figure 3. The void volume of this column was such that Blue Dextran eluted at fraction 43. Recovery of radioactivity from the column was greater than 85% of the applied count rate. Free $Na^{125}I$ eluted at fraction 160 (data not shown).

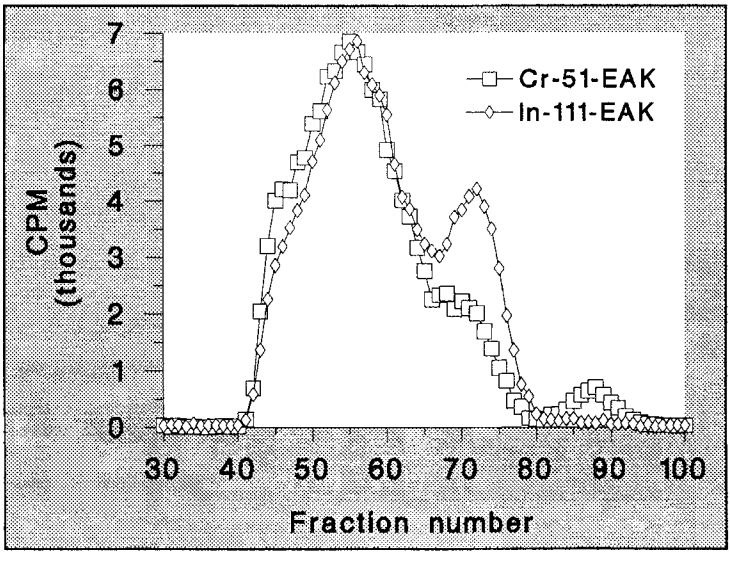

Figure 3 S-300 elution profiles of EAK (polypeptide #4) labelled with ^{51}Cr or ^{111}In by DTPA chelation. Same S-300 column as in Figure 2. Recovery of radioactivity from the column was greater than 70% of applied count rates. Free $^{111}InCl_3$ or free $^{51}CrCl_3$ added to PBS and run on this column eluted as sharp peaks at fraction 90 (data not shown). There is some ^{51}Cr from ^{51}Cr labelled EAK at this point, but this accounted for less than 3% of the total.

with ^{111}In or ^{51}Cr. There was little evidence of the presence of free radionuclides in the labelled preparations, since high proportions of the applied counts eluted well before the position of elution of free radionuclides (Figures 1-3).

S-300 gel permeation chromatography of D-KAK, labelled with ^{125}I, ^{111}In, or ^{51}Cr was not feasible, since this material adhered non-specifically to this gel permeation medium. This is no doubt due to the polycationic nature of D-KAK, allowing reaction with the small number of free carboxyl groups on this cross linked allyl dextran.

Blood clearance of radiolabelled polypeptides

Previous studies examine the blood clearance profiles of a range of polypeptides labelled with ^{125}I by reaction with pre-labelled Bolton and Hunter reagent [17], and identified EAK as having the greatest blood survival. Although we have previously reported labelling of EAK with ^{111}In, a formal comparison of the blood survival of ^{125}I and ^{111}In labelled material had not been carried out previously. Test 1 (Table 2) shows the result of such a comparison. EAK (polypeptide #1, Table 1) was reacted with DTPA anhydride and ^{111}In labelled, or it was directly labelled with ^{125}I labelled Bolton and Hunter reagent. Following injection into mice, the blood survivals of the ^{111}In and ^{125}I were virtually identical, so that their AUC's were 316 and 339 percent dose:hours respectively.

Figure 4 shows blood survival curves of the amphoteric EAK (polypeptide #2, Table 1) labelled with ^{125}I or ^{111}In, and its cAD conjugate (polypeptide #3) labelled with ^{125}I, ^{111}In or ^{51}Cr. Here ^{125}I labelling was carried out by iodination of Bolton and Hunter reagent previously conjugated to the polypeptides. All five labelled materials showed the same good survival in blood. When quantified as areas under the time activity curves (AUC), values were within the range 182-251 percent dose:hours (Test 2, Table 2). In contrast, ^{125}I labelled polycationic D-KAK was cleared rapidly from the blood, the AUC being only 31 percent dose:hours (Figure 4 and Test 6, Table 2).

Figure 5 shows blood survival curves of polypeptides labelled with radiometals. EAK showed prolonged blood survival, whether labelled with ^{111}In or ^{51}Cr. D-KAK, labelled with

Table 2 Blood kinetics and biodistribution of radiolabelled branched chain polypeptides

Test number	Polypeptide		Label	AUC in blood 0–4 hrs (%dose:hrs±SD)		Retention at 4 hours (%dose±SD)	Percent dose (±SD)/gram of:–						
	Material	Polymer #					Blood	Spleen	Intestine	Kidney	Liver	Lung	Carcass
1	EAK	1	I–125	338.7±10.3	–	55.1±1.6	34.0±2.1	10.0±0.5	1.4±0.1	5.3±0.3	7.7±0.4	10.4±2.3	2.0±0.1
	EAK	1	In–111	315.7±12.7	–	67.9±3.6	28.1±2.8	15.2±1.8	1.1±0.1	32.5±3.2	17.8±0.5	6.8±0.6	2.0±0.3
2	EAK	2	I–125 *	213.7±17.8	Fig 4	44.3±1.0	16.5±1.1	2.1±0.2	2.1±0.2	5.1±0.2	3.0±0.1	4.5±0.3	2.0±0.2
	EAK	2	In–111	250.7±9.1	Fig 4	57.0±3.0	19.1±0.6	4.1±0.1	1.9±0.4	40.3±6.6	9.2±0.7	5.8±0.6	1.5±0.1
	EAK–cAD	3	I–125 **	180.3±2.7	Fig 4	40.1±2.6	12.4±1.0	2.1±0.4	2.6±0.5	5.6±1.1	2.8±0.6	3.7±0.5	1.8±0.2
	EAK–cAD	3	In–111 **	188.1±2.2	Fig 4	51.8±1.4	14.4±1.3	4.7±0.5	1.6±0.3	23.4±3.0	10.0±1.1	4.4±0.7	1.9±0.2
	EAK–cAD	3	Cr–51	181.9±4.7	Fig 4	49.9±0.7	11.8±1.1	6.6±0.7	1.3±0.1	10.6±1.6	17.7±1.3	3.0±0.3	1.7±0.1
3	EAK–cAD	3	I–125	164.1±7.7	–	38.0±2.9	10.7±0.4	1.6±0.5	2.8±0.6	5.3±0.3	2.3±0.1	3.3±0.4	1.4±0.1
	EAK–cAD	3	In–111	242.7±6.4	–	57.0±2.6	14.7±1.1	5.2±0.4	1.9±0.2	20.0±2.5	9.5±0.7	5.1±0.5	2.4±0.2
4	EAK	4	In–111	182.0±2.6	Fig 5	41.3±0.4	11.1±0.1	3.3±0.3	1.1±0.2	10.2±0.4	8.3±0.1	3.9±0.1	1.7±0.1
	EAK	4	Cr–51	146.4±5.1	Fig 5	42.3±1.0	9.6±0.2	4.5±0.4	0.9±0.2	6.5±0.3	18.1±0.2	3.5±0.1	1.3±0.1
5	EAK	4	In–111 *	158.4±11.8	–	44.4±3.1	11.6±0.8	3.3±0.4	1.8±0.4	11.5±0.7	9.4±1.2	3.3±0.2	1.6±0.3
6	D–KAK	5	I–125 ***	30.7±1.5	Fig 4	76.6±4.1	1.2±0.1	43.6±5.0	5.3±0.5	28.9±5.3	19.7±1.3	26.0±2.9	1.5±0.1
	D–KAK	5	In–111	37.1±2.0	Fig 5	85.0±1.6	2.0±0.1	31.6±4.4	4.7±0.2	45.7±1.6	43.3±2.2	14.4±0.8	2.1±0.1
	D–KAK	5	Cr–51	33.8±3.7	Fig 5	72.0±1.2	1.5±0.2	71.1±10.8	1.4±0.2	10.0±0.4	36.2±2.6	62.1±4.4	1.5±0.1
7	D–KAK	5	In–111 ***	33.9±1.7	–	96.6±4.8	1.3±0.1	64.0±8.6	4.9±0.5	48.1±8.6	36.7±2.6	20.1±2.3	1.3±0.1

* ** *** Labelled materials admixed before injection and count rates determined simultaneously. Four mice in each group.

either radiometal, was cleared rapidly from the circulation. Tests 4 and 6 (Table 2) shows the calculated AUC's for these clearance curves.

The small intra-test differences in the blood clearance profiles of polymers with or without cAD, and with different radiolabels, seen in Figures 4 and 5, were no greater than inter-test differences with any one polymer with any one radiolabel. Table 2 shows tests with three of the polypeptides which were labelled and tested in vivo more than once. In Test 2, with ^{125}I labelled EAK-cAD, its AUC was 180 percent dose:hours, and in Test 3 it was 164 percent dose:hours. The same material labelled with ^{111}In gave AUC's of 188 (Test 2) and 243 (Test 3). ^{111}In labelled EAK gave AUC's of 182 in Test 4 and 158 in Test 5, and D-KAK values of 37 in Test 6 and 34 in Test 7.

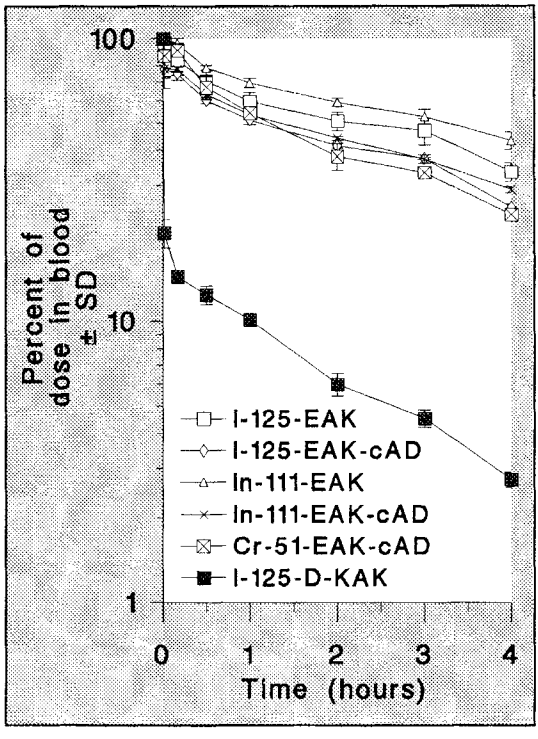

Figure 4 Blood clearance profiles after intravenous injection into mice of amphoteric polypeptide EAK (polypeptide #2) labelled with ^{125}I or ^{111}In, and its cAD conjugate (polypeptide #3) labelled with ^{125}I, ^{111}In or ^{51}Cr, and of polycationic D-KAK (polypeptide #5) labelled with ^{125}I. Four mice/group.

Organ distribution of the radiolabels from the polypeptides

Table 2 shows the levels of the radiolabels in the organs of mice in which blood clearance tests had been carried out, when they were killed four hours after injection of labelled polypeptides.

With EAK's, and cAD conjugates, the total body retention of any radiolabel from any one polypeptide were similar, although this tended to be higher for ^{111}In than for ^{125}I (Tests 1-3). This was probably due to kidney, liver and spleen retaining lower levels of ^{125}I than of the radiometal ^{111}In.

Although Tests 1-3 showed that ^{111}In was retained better in liver, kidney and spleen than was ^{125}I, the same distribution was not seen with the other radiometal, ^{51}Cr. Tests 2 and 4 (Table 2) showed that with any one polypeptide or its cAD conjugate there is a higher retention of ^{51}Cr than ^{111}In in the liver, but a lower retention in the kidney.

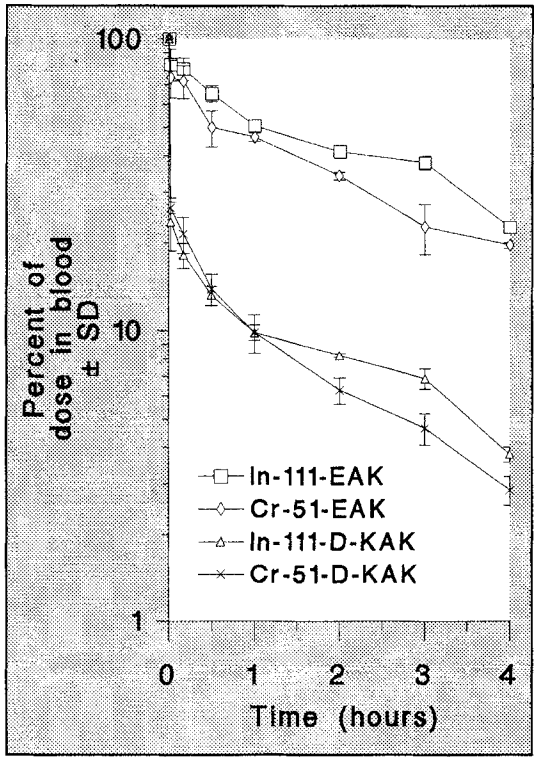

Figure 5 Blood clearance profiles after intravenous injection into mice of amphoteric polypeptide EAK (polypeptide #4) and of polycationic polypeptide D-KAK (polypeptide #5) after labelling with ^{111}In or ^{51}Cr. Four mice/group.

It is difficult to be sure of the cause of the different patterns of biodistribution of the different radionuclides used to label EAK, but it is most likely due to differences in biological handling of radionuclides in particular organs, rather than to true differences in biodistribution of polypeptides with the different labels. Certainly it is well known that with antibodies labelled with radioiodine, by oxidative incorporation into their tyrosine moieties, or with ^{111}In by DTPA chelation, there is a more prolonged retention of the radiometal in many organs, particularly liver, kidney and spleen. This is due, at least in part to lysosomal retention of ^{111}In-DTPA-lysine conjugate, presumably released during breakdown of labelled proteins [24].

With ^{125}I labelled polycationic polypeptide D-KAK, there were much higher levels of radiolabel in spleen, liver, kidney and lung (Test 6, Table 2) than were had been seen with EAK, suggesting that these were organs involved in its rapid clearance. There were also much higher levels of ^{111}In and ^{51}Cr from labelled D-KAK in these organs (Tests 6 and 7, Table 2) than had been seen with EAK, again suggesting that these are principal sites of the rapid clearance of D-KAK from the blood. Greater retention of ^{111}In than ^{51}Cr in the kidney, seen with EAK, was also seen with D-KAK.

CONCLUSIONS

This study has shown that it is possible to label these two classes of branched chain polypeptides with gamma emitters such as ^{125}I and ^{51}Cr, suitable for simple biodistribution studies, and with others suitable also for gamma scintigraphy (^{123}I, ^{131}I, ^{111}In). It was possible to prepare DTPA conjugates for radiometal labelling, or Bolton and Hunter conjugates for radioiodine labelling, which were stable in solution, indeed stable for several years. Obviously this can facilitate labelling, and in particular it enables labelling with ^{131}I and ^{123}I, which are not routinely available as iodinated Bolton and Hunter reagent. In vitro, at least as assessed by gel permeation chromatography, and in vivo, at least as assessed by survival in the circulation, there were virtually no differences between preparations labelled with the various radionuclides. There were however differences between patterns of organ distribution of the three different radionuclides from any one polypeptide. The indication is that the radiometals are retained better than radioiodine at the sites of clearance of the

polypeptides, suggesting that such radiometals may be the radionuclides of choice for determining any slow non-specific accumulation of such synthetic macromolecular polymers at sites of pathological lesions, such as any enhanced permeability and retention in tumours, which has been reported with other macromolecules [20,25]. Of the radiometals examined in the present study, only ^{111}In is suitable for gamma camera imaging. Even this is not ideal, and, particularly for clinical studies, ^{99m}Tc would be more suitable. The feasibility of labelling these polypeptides with ^{99m}Tc is currently under investigation.

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