The use of redox polymers in labelling procedures of proteins and peptides with ^{99m}Tc

I. Properties of redox polymers and technique of labelling

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Abstract

BACKGROUND: Using a polymer-analogue reaction, redox polymers with a dextran matrix to anchor the end $\alpha(\beta)$-alanine--N, N′-diacetate group converted into the Sn^{2+} cycle have been developed for the labelling of proteins, peptides, and labile compounds with ^{99m}Tc. The reaction kinetics and the labelling efficiency of ^{99m}Tc depend primarily on the degree of dextran matrix cross-linking and the qualitative nature of the redox polymer end groups.

METHODS: Preparation for labelling takes place directly in basic protein and peptide solutions without adjusting pH or adding other adjuvants. Prior to the final modification into kit form, the redox polymers are removed by ultrafiltration.

RESULTS: The results of labelling of model compounds (aspartic acid, cysteine) with ^{99m}Tc at various solution pH values clearly show that, at pH values over 6.0, it will be primarily the free end SH groups that will serve as binding sites for the proteins and peptides. To label proteins and peptides, we selected a redox polymer with G-25 cross-linking, which allows the achievement of a radiochemical purity over 95% and high stability of the labelled compounds.

CONCLUSION: The method of radiolabelling compounds with ^{99m}Tc based on redox polymers was developed mainly to overcome the problems faced when using other conventional methods in the labelling of sensitive compounds.

Key words: redox polymers, technetium (^{99m}Tc), labelling procedure, chelating groups

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Introduction

In lower oxidation states, technetium possesses a strong tendency to form complexes, in this way the oxidation state stabilises (1,2). Comparing the electronegativity of some elements (O 3.5, N 3.0, S 2.5) to that of Tc, which is as low as 1.35, it is evident that, in organic molecules, Tc will seek sites with the greatest electron density in the first place. When labelling the compounds with ^{99m}Tc, it is necessary to take into account — in addition to steric factors — donor characteristics of group, the resonance effect, group basicity, etc. and, also, the redox potential. Oxidation-reduction polymers, or „redox polymers" (3) are cross-linked insoluble compounds with a high molecular weight that can transfer or „exchange" electrons with reactive ions or molecules. The advantages of using redox polymers as reducing agents for technetium in labelling procedures outweigh those of stannous chloride. The use of stannous chloride as a reducing agent is limited to a low range pH (1–2.6), when the pH of the solution is raised. While stannous chloride tends to hydrolyse (4), redox polymers can be used over a wide range of pH values. This is an advantage over conventional methods because many organic ligands are found to decompose readily at low pH values, making it impossible to label them using a tin cation as a reducing agent for pertechnetate.

The basic requirements for the use of redox polymers in labelling procedures with ^{99m}Tc are as follows: suitable potential, chemical stability (resistance against changes in pH and temperature), sufficient reaction rate and redox capacity.

Our method is clearly designed to label labile structures. It is the question of compounds, not only of proteins and peptides but also of structurally proper types of hormones, enzymes, vitamins, etc. The characteristic arrangement of protein and peptide mole-

cules conformation predetermines the especial significance of steric factors for the stability of arising complexes and so it introduces the possibility of certain specificity in the bond (5).

According to our conceptions (6) the binding centre in these molecules largely forms (for pH values of up to 6) the end SH groups. In molecules with a double bond on S, the position of the group, which is capable of yielding the H ion due to the resonance effect is probably also of importance. Introducing SH groups markedly increases the stability of complexes. The end SH groups in these molecules can be generated of double bond S, i.e. with UV irradiation (7–9).

Materials and methods

Synthesis of redox polymers with IDA functional groups

Chemicals

L-Cysteine hydrochloride anhydrous (CYS), Fluka, MicroSelect
L-Aspartic acid (ASP), Merck
Sephadex (G-10 – G-200) fine, Pharmacia, Uppsala, Sweden (a)
99.8 % ethanol (b)
10M solution of NaOH (c)
Disodium salt of iminodiacetic acid (IDA), Koch Light (d)
2,3-dibrompropionic acid, Koch Light (e)
Acetone redistilled (f)
Concentrated acetic acid
Water for injection
All chemicals utilised in the experiment were of analytical grade.

Procedure

The synthesis of redox polymers with IDA functional groups from the dextran matrix involves two main steps, the attachment of the functional groups to the polymer matrices and the binding of the Sn^{2+} cation to the functional group.

Step 1. The dextran matrix (a) is added to a basic medium (c, b) with iminodiacetic disodium monohydrate (d) and left to mix, thereby forming a homogeneous reaction environment. With respect to the chemical reactivity of the substrate and the reactants, it was necessary to perform the attachment of the function groups to the dextran under conditions whereby degradation and destruction of the particles were prevented. To this effect, the following method was used: the flask containing the compound was rotated using an electromagnetic motor, which held the cooling system in place. A bifunctional agent (e) in a suitable organic solvent (f) was then added to the reactants. This system of reactants was left to react under continuous mixing in an oil bath at a temperature of 78° C. The product was then rinsed with a solution of 125 parts of ethanol, 125 parts of water and one part of 1M HCl (approximately one litre). The suspension in distilled water was then poured into a column where it was rinsed with distilled water three to four times. Finally, it should be rinsed three times with about 20 ml of concentrated ethanol and dried under nitrogen gas.

Step 2. 8.86 mM solution of dihydrate tin chloride in Clark-Lubbs buffer pH 2 under nitrogen gas was then diluted 1:1 with the above buffer solution. The resulting solution was then poured over 2g of polysaccharide microspheres in the column and then left to drip in a nitrogen gas atmosphere. This was followed by rinsing with injection water, buffer, distilled water and, finally, ethanol. The redox polymers were left to drip in an atmosphere of nitrogen gas.

Labelling of compounds with ^{99m}Tc

The redox polymer was mixed with the solution of the ligand at room temperature for 8–12 hours. The reaction was then terminated by filtering through a Millipore filter (0.22μm) into a sterile vials and the eluate $^{99m}TcO_4^-$ of the desired activity was added. The mixture was incubated in boiling water (in case of aspartic acid labelling) or at room temperature (labelling of cysteine or other proteins) for 10–15 minutes.

Methods of analytical control

Radiochemical purity

The labelling efficiency was evaluated by means of paper chromatography (Whatman 1 or Whatman 3 using saline as a solvent) and column chromatography (column of 30 x 1 cm filled with Sephadex G-50. Sample amounts of 50 μl were applied and eluted by saline as a mobile phase).

Results

The basic philosophy of preparing kits using redox polymers is based on our data obtained in experiments with ^{99m}Tc labelling of model compounds. Using redox polymers, we labelled cysteine — as a typical representative — with end SH groups and aspartic acid with a combination of two carboxylic acid groups and one amino acid group. The results of radiochemical purity related to pH are shown in Figure 1.

When preparing kits of proteins and peptides for ^{99m}Tc labelling, we used redox polymers with a dextran skeleton with cross-linked epichlorhydrine with IDA-type end groups. The surface area of redox polymers was scanned by a direct reflection electron microscope and pictures were taken using a Stereoscan S 600, CSIL Cambridge. The contour-like surface shown in Figure 2 is necessary for a high kinetic rate.

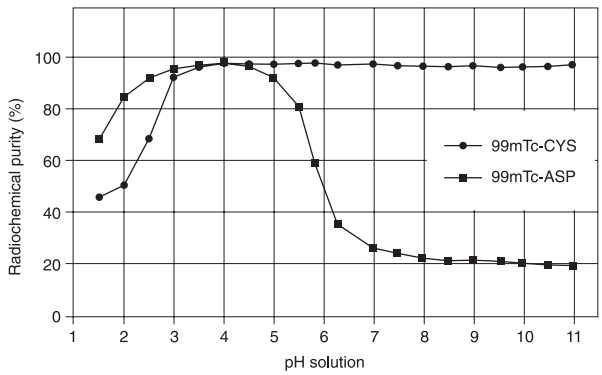

Figure 1. Dependence of radiochemical purity of ^{99m}Tc-CYS and ^{99m}Tc-ASP on pH.

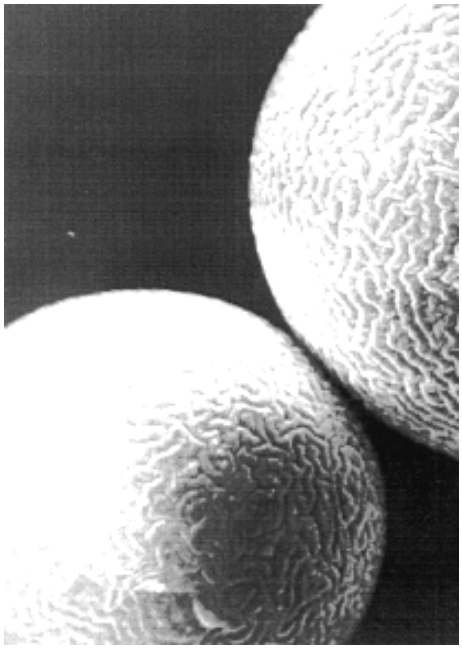

Figure 2. Images of redox polymers (Mx5000).

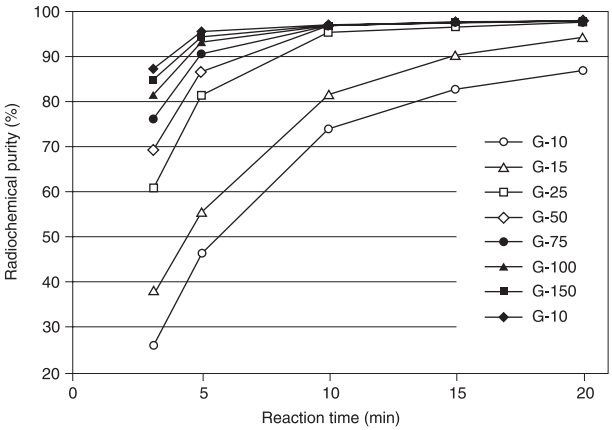

Figure 3. Dependence of radiochemical purity of ^{99m}Tc-CYS on cross-linking of dextran matrix of redox polymers.

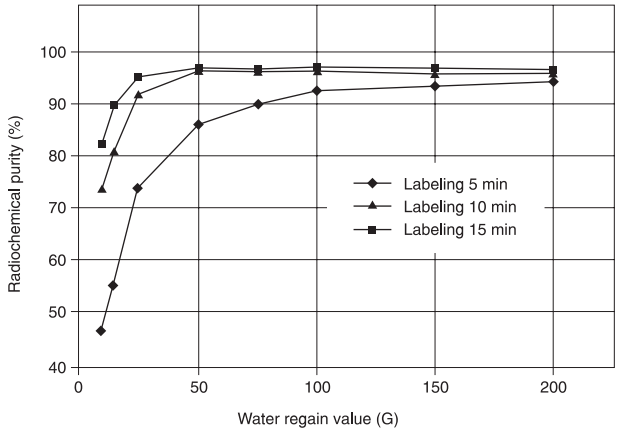

Figure 4. Relationship between radiochemical purity of ^{99m}Tc-CYS and water regain value of redox polymer.

Using a model system, we monitored the reaction kinetics of ^{99m}Tc-CYS formation dependent on the degree of cross-linking of the dextran skeleton of redox polymer. The results are shown in Figure 3.

The relationship of radiochemical purity of ^{99m}Tc-CYS on the degree of redox polymer dextran skeleton cross-linking is shown in Figure 4.

Discussion

It is evident from the course of the curves in Figure 1 that the highest radiochemical purity of labelled aspartic acid (over 97%) is in the range of pH 3.5–4.5 with a relatively steep fall at highest pH values whereas, with labelled cysteine, the value is over a substantially broader range of pH 3.5–11 (not investigated over pH of 11). This is due to the high affinity of reduced technetium to the end SH groups. The difference can be made use of when preparing kits for protein or peptide labelling, especially those with a pH in the range of 6.5–8 but, also, with higher pH, provided the proteins or peptides are stable. With the above pH values, specific labelling essentially occurs to the end SH groups, and the labelled proteins and peptides show high stability.

The redox polymers with a dextran skeleton are insoluble polyelectrolytes capable of reverse oxidation or reduction. They are microsphere-shaped, measuring 20–80μm in diameter and can be readily removed from the reaction mixture by ultrafiltration. Their efficacy and reaction kinetics depend primarily on two basic parameters:

a) diffusion rate at the phase interface, with the rate affected by the degree of cross-linkage,

b) qualitative nature of end function groups.

Ad a) It is known that solid surfaces play an important role in adsorption, contact catalysis, corrosion, and rate of chemical reaction. The degree of cross-linking is expressed as „water regain value G", whereby the degree of cross-linking declines as the values increases. It is clear from the kinetics of ^{99m}Tc-CYS formation that redox polymers with the highest degree of cross-linking, i.e. G-10 and G-15 do not achieve radiochemical purity greater than 95% as long as 20 minutes after labelling. All other redox polymers meet this condition as early as 10 minutes after labelling.

Use of redox polymers with the highest degree of cross-linking (G-10 and G-15) does not result in the required radiochemical purity over 95% within a reasonable period of time. By contrast, redox polymers with the lowest degree of cross-linking (G-150 and G-200) meet this requirement after 5 minutes of labelling. To prepare kits of proteins and peptides for ^{99m}Tc labelling, we chose G-25 cross-linking. However, it should be noted that loss of protein and peptide solutions increases during incubation with a redox polymer as the degree of cross-linking decreases. For instance, with the lowest degree of cross-linking, G-200, these losses may be as high as 30%; they are due to increased tendency to swelling of gel-like redox polymers. The loss of solution volume can be eliminated by incubating the protein and peptide solutions with a redox polymer in a micro-column, especially with costly compounds; this will be the subject of future experiments.

Ad b) The stability and radiochemical purity of labelled compounds is largely influenced by the chemical structure of the redox polymer end function groups. Conversion of a redox polymer is effected by entering the polymer into the Sn cycle, or that of other metallic cations with an appropriate redox potential. Charged

polymer derivatives with end $\alpha(\beta)$-alanine-N, N'-diacetate groups have been shown to be more effective than charge-free iminodiacetate derivatives. A schematic representation of the structure is shown in Figure 5.

The redox polymers of this type are characterised by a number of physical and chemical constants. During synthesis, two types of structures — in terms of position of the carboxyl group (i.e., positions α or β) — form in the alanine linking chain. Unlike commercial polymers (10,11) converted to the redox polymers, this arrangement exerts a strongly positive effect both on the radiochemical purity and stability of the labelled compounds.

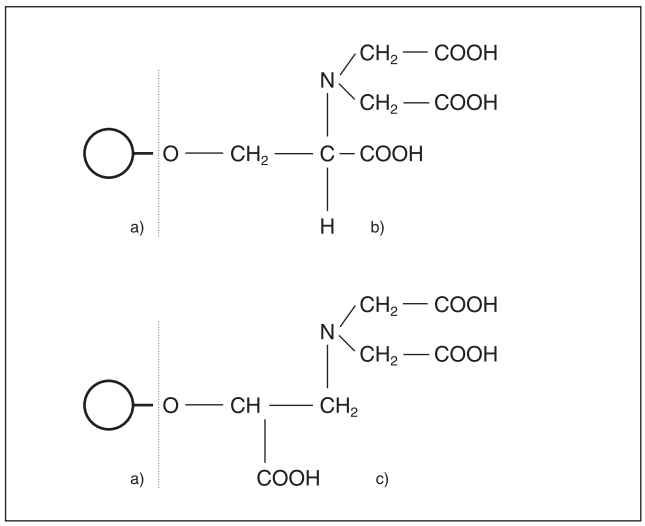

Figure 5. Structure of the end functional groups of redox polymer.
a) dextran skeleton, b) α-alanine-N, N'-diacetic group,
c) β-alanine-N, N'-diacetic group.

Conclusions

Redox polymers of dextran matrices used in the labelling procedure were synthesised by a polymer-analogous reaction and a tin cation was attached to the functional group by a chelate bond forming the most suitable reductant. The rate of formation of the ^{99m}Tc-complex and its radiochemical purity are highly dependent on the quality of the end groups of the redox polymer, on the number of pores on the surface and the swelling of the microspheres.

The method of radiolabelling compounds with ^{99m}Tc based on redox polymers was developed mainly to overcome the problems faced when using other conventional methods in the labelling of sensitive compounds.

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