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Labeling, characterization, and in vivo localization of a new ^{90}Y-based phosphonate chelate 2,3-dicarboxypropane-1,1-diphosphonic acid for the treatment of bone metastases: Comparison with ^{99m}Tc–DPD complex

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Abstract—The goal of this investigation was to examine the possibilities for yttrium-90-labeling of the 2,3-dicarboxypropane-1,1-diphosphonic acid (DPD), which is currently labeled with technetium-99m and as a ^{99m}Tc–DPD clinically used as bone imaging agent. Analysis of the complex enclosed the radiochemical quality control methods, biodistribution studies, as well as the determination of pharmacokinetic parameters. The biological behavior of complexes ^{90}Y–DPD, ^{99m}Tc–DPD and ^{90}Y-labeled DPD-kit formulation [^{90}Y–(Sn)–DPD] in animal model was compared. The labeling conditions were standardized to give the maximum yield, which ranged between 93% and 98%. The examined ^{90}Y complex could be easily prepared, with an outstanding yield and was also found to be very stable for at least 10 h after ^{90}Y-labeling. Protein binding value was 4.6 ± 0.7% for ^{90}Y–DPD complex and the complex possess a hydrophilic character. The satisfactory results of ^{90}Y–DPD biodistribution in healthy test animals were obtained; the uptake in the bone was 11–13% ID/g after 24 h depending on the pH value during the preparation. With high skeletal uptake, a minimum uptake in soft tissues and rapid blood clearance the ^{90}Y–DPD complex proved to be an excellent candidate for targeting tumor therapy.
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1. Introduction

In recent years, the use of compounds labeled with radionuclides for the therapeutic treatment in the medical field has grown considerably. Such radiopharmaceuticals are increasingly used for cancer therapy, palliation of pain caused due to bone metastasis and for the treatment of rheumatoid arthritis.[1–3] Primary tumors (breast, lung, prostate, kidney, etc.) frequently metastasized and the bone is one of the most common sites of this metastasis in cancer patients, while the bone pain is the most prominent symptom associated with bone metastases. The pain becomes progressively severe as the disease advances. Targeted radionuclide therapy involves the specific deposition of β-emitting radionuclides in malignant tumor via labeled ligands that specifically bind to tumor cells to reduce the amount of injury to normal tissue and is found to be an effective treatment for the palliation of pain.[4–6] Because of that several classes of chelated ligands are being evaluated for therapy, and simple effective methods are available.[7–9] The therapeutic radiopharmaceuticals, based on electron emission of particular radionuclides and isotopes decaying by the emission of β-particles, are preferred in most of these applications. Nowadays, ^{90}Y is a radioisotope widely used for therapy and obtained from ^{90}Sr as a high yielded fission product. Yttrium-90 is a high energy β^- particle emitter, with $E_{\max\beta^-} = 2.27$ MeV and $t_{1/2} = 64.4$ h.[10–12] Furthermore, the considerable path length in tissues of its β^- particles ($r_{95} = 5.9$ mm) represents a major advantage in solid tumors. Higher energy and hence longer particle range in tissues provide the ability to treat larger tumors.[13,14] The lack of gamma radiation makes patient treatment feasible. Thus it has gained acceptance in clinical practice.

For the detection and evaluation of bone metastasis, ^{99m}Tc labeled phosphonates compounds has been widely accepted for bone scintigraphy, because of its high sensitivity and easy evaluation of the whole skeletal system. Methylenediphosphonate (MDP), dicarboxypropane diphosphonate (DPD), hydroxyethylidine diphospho-

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nate (HEDP), and ethylenediamine-tetramethylene phosphonate (EDTMP) labeled by ^{99m}Tc are clinically used.[15–18] The bone uptake mechanisms of phosphonates compounds are ion exchange and chemisorption in the inorganic matrix of the bone, which consists of ionic hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Phosphate groups of the surface of the bone matrix react with the free PO_3H_2 groups of the used phosphonates ligand coordinated to radioactive metal ion. Thus, this ion exchange reaction results in the adsorption of the metal ion—activity on the bone matrix. This process occurs in the healthy bone, but accumulation is higher at sites that are characterized by the increased bloodstream and increased ossific activity (osteoblast function). Phosphonic acid derivatives labeled with radionuclides emitting β-particles are found to be effective palliative agents for the treatment of bone pain.[19–23]

A major concern with ^{90}Y-labeled substances is the selection of a chelate with sufficient thermodynamic and kinetic stability to prevent in vivo loss of radiometals from the complex because dissociated $^{90}Y^{3+}$ rapidly accumulates in the bones, delivering an undesired amount of radiation to the radiosensitive marrow.[24] The substance 2,3-dicarboxypropane-1,1-diphosphonic acid labeled with ^{99m}Tc (^{99m}Tc–DPD) is used as bone-seeking radiopharmaceutical for skeletal imaging to detect the pathological foci.[25] Also this substance as tetradentate ligand (with two phosphonates and two carbohylic groups) is as effective chelating agent. Until now it is radiolabeled only with ^{99m}Tc.

In the present study, we investigated the optimal ^{90}Y-labeling conditions for DPD by changing several reaction parameters (concentrations of ligands, pH, temperature, reaction times, etc.). The in vitro binding to human serum albumin, lipophylicity and the stability of this complex was also performed. The biological properties were done in healthy white male Wistar rats.

Thermodynamic stability of radiopharmaceuticals is of the crucial interest, because the loss of radiometal may result in accumulation of radioactivity in non-target organs. Therefore, the selected chelator must form a complex with high thermodynamic stability to retain its chemical integrity in competition with natural chelators present in a blood stream. Chemical stability of compounds may be judged according to molecular energy data. Application of molecular modeling as a computation tool is useful for preliminary theoretical analysis of chemical compounds without resorting to laboratory testing. The molecular modeling studies have been carried out for structural analysis of the complexes using software HyperChem™ release 6.03, version for Windows.[26–29]

2. Results

2.1. Radiochemistry

A series of experiments were carried out in order to determine the optimal ^{90}Y-labeling conditions for DPD substance. The labeling efficiency was measured by a combination of ascending paper, thin-layer chromatography, and HPLC methods. The HPLC radiochromatographs of the labeling complex showed satisfying separation of free ^{90}Y from ^{90}Y–DPD: retention times were 4.32 and 23.12 min, respectively. The TLC-silica gel and paper Whatman No. 1 strips were estimated as an appropriate stationary phase for the assay. For finding out a suitable mobile phase, different organic solvents in various ratios were screened. Radiochemical purity was then determined using chromatography with appropriate stationary and mobile phases. The paper chromatography with pyridine–acetic acid–water (5:3:1.5, V/V) as the mobile phase showed excellent resolution of components, with R_f value of the complex around 1.0 and the free ^{90}Y retained at the origin ($R_f = 0.0$). Also, the other chromatography systems that were applied showed satisfactory results. The obtained results are presented in Figure 1.

The correlation between the concentration of DPD ligand and the labeling yield is shown in Figure 2. It

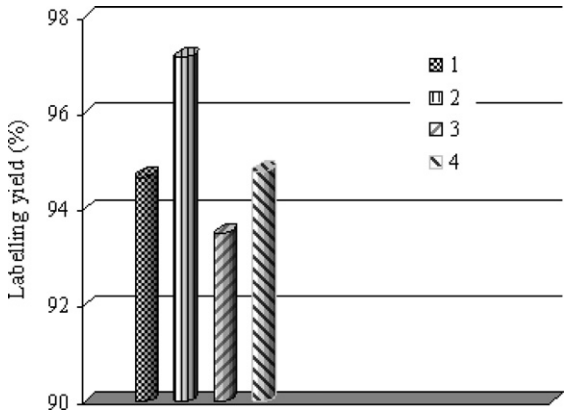


Figure 1. Determination of labeling yield for ^{90}Y–DPD with different chromatographic methods (30 min at 95 °C): (1) Whatman No. 1/0.1 M phosphate buffer pH 6.5, (2) Whatman No. 1/pyridine–acetic acid–water (5:3:1.5, V/V), (3) silica gel 60/ethyl acetate–ethanol (1:1, V/V), (4) HPLC.

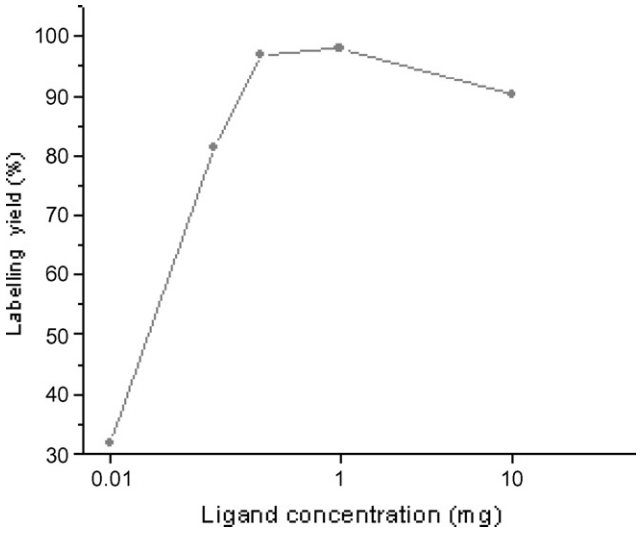


Figure 2. Effect of the ligand concentration on the ^{90}Y–DPD labeling yield (30 min at 95 °C).

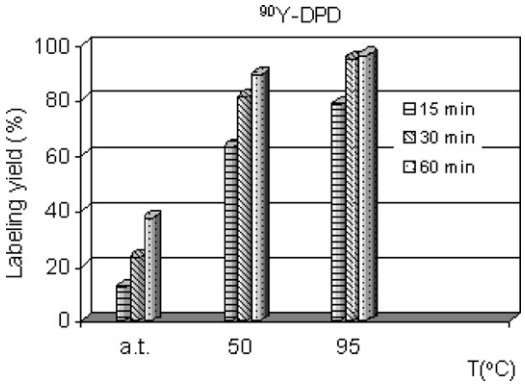


Figure 3. Effect of temperature and heating time on labeling yield for ^{90}Y–DPD.

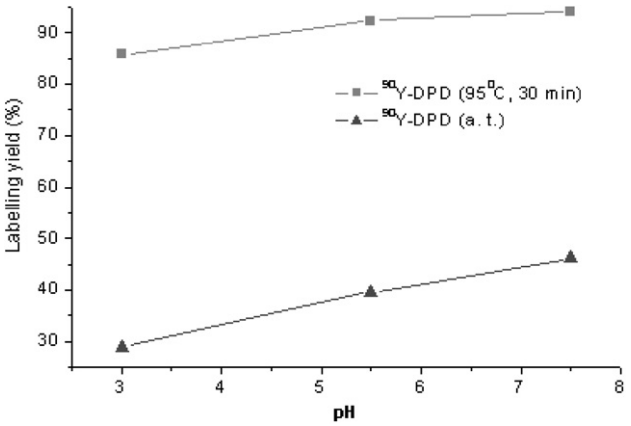


Figure 4. Effect of solution pH value and temperature on the labeling yield for ^{90}Y–DPD.

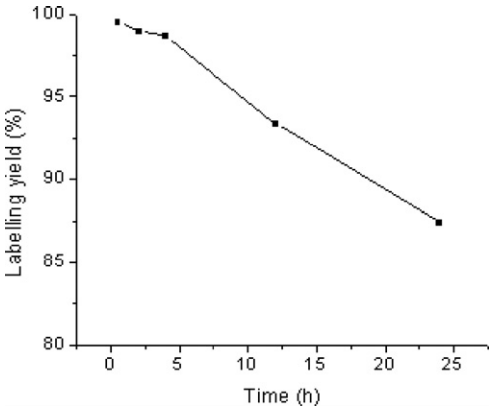


Figure 5. Stability study of ^{90}Y–DPD complex (prepared at 95 °C for 30 min) stored at various intervals of time at ambient temperature.

was observed that at the minimum concentration of 0.1 mg/ml (~0.35 μmol/ml) of DPD ligand labeled with trace amounts of ^{90}Y (~0.337 nmol) during the reaction time of 30 min at 95 °C, for ligand to metal ratio of 1038:1, significant labeling of about 82% was achieved. As the concentration of ligand increased, labeling was required near quantitative yield; about 98% of ^{90}Y–DPD complex was obtained.

The influence of temperature on the labeling yield was also obvious. When the labeling of the investigated ligand with ^{90}Y was performed at ambient temperature (a.t.), it was found to be poor as compared to the yield obtained by heating the reaction mixture (in a water bath during different times). The labeling efficiency of the ^{90}Y–DPD preparation at a.t. was between 25% and 45%. When the labeling of the DPD with Y-90 was carried out at higher temperatures (50 and 95 °C) for different time intervals (15, 30, and 60 min), there were significant increases in the labeling yield (Fig. 3). The samples heated at 95 °C for 30 min provided the highest labeling yield. So, all further experiments (protein binding, lipophilicity, biodistribution study) were performed with heated samples prepared in the same manner. After cooling the samples down to ambient temperature, HPLC purification was performed.

Concerning the influence of pH values on the labeling yield it seemed that the values did not have any higher effects on the labeling yield, especially in the heated samples. Thus for ^{90}Y–DPD heated samples, the yield of 92.3% and 94.1% at pH 5.5 and at pH 7.5 was obtained, respectively (Fig. 4).

2.2. Stability studies

The stability of the complex varied depending on the labeling conditions. Thus, the complex prepared at ambient temperature showed decomposition within 4 h. The decomposition rate for complex prepared at higher temperatures varied depending on the temperature and the reaction time. The rate of complex decomposition decreased when the temperature and heating time increased (>15 min). The optimum heating time for attaining maximum stability was 30 min at 95 °C. The results of the stability study for the ^{90}Y–DPD complex prepared in the mentioned condition and then stored at a.t. during different times are presented in Figure 5. It can be seen that there is no significant decrease in radiochemical purity for the ^{90}Y–DPD complex throughout the period of 10 h.

2.3. Serum stability studies

The stability of ^{90}Y–DPD in human serum was assessed by measuring the release of ^{90}Y from the complex at 37 °C over a 10-day period. The serum stability result of ^{90}Y–DPD showed that the complex was quite stable at the studied conditions for more than 10 days. No significant dissociation of activity from the complex was observed. The percentage of ^{90}Y released from this complex at 10 days was <2.0%.

2.4. Protein binding and lipophilicity studies

The protein binding results of ^{90}Y–DPD as well as those for ^{99m}Tc–DPD (both prepared at pH 7.5), determined by the TCA precipitation method, have shown that during the incubation time of 1 h only 3.8–5.2% (4.6 ± 0.7% for $n = 5$) and 48–51% (48.6 ± 1.9% for $n = 5$) was bound to 12% HA, respectively.

The lipophilicity of ^{90}Y–DPD and ^{99m}Tc–DPD was determined using the following method prescribed below. The results of lipophilicity measurements of these complexes showed that all radioactivity remains in the aqueous phase, thusly the distribution coefficient is approximately zero, namely complexes are hydrophilic. No change in extractability with pH was observed.

2.5. In vivo experiments

Biodistribution studies of the ^{90}Y–DPD complex purified by HPLC and diluted with saline were performed in an animal model—healthy male Wistar rats. Retention of this complex in animal model was observed up to 24 h.

Following the intravenous injection of an aqueous solution of $^{90}YCl_3$, adjusted to pH 7.5 with 0.1 M NaOH solution, through the tail vein of normal rat (Fig. 6.), radioactivity was found to distribute mainly in bone at 1 h (6.02% ID/g) and at 24 h (4.32% ID/g). The radioactivity of bone slowly decreased with the time. The uptake in the bone was calculated from the activity noted in femur per gram. The other organs uptake at 1 h post injection (pi) was cleared within 24 h pi except in the muscle. The bone/blood ratio was 6.81 and 98.29, but the bone/muscle ratio was 16.23 and 13.78 at 1 and 24 h pi, respectively. Those results pointed out longer retention of ^{90}Y in the femur, the blood activity was not detected after 24 h pi, but there was very slow clearing activity from the muscles.

The comparing biodistribution study results of ^{90}Y–DPD complex, prepared at different pH, in selected organs of the healthy Wistar rats are presented in Figure 7. These results showed varying degrees of bone uptake in rats, which was dependent on preparation pH and the time post injection. The biodistribution of DPD labeled with ^{90}Y at pH 7.5 showed higher bone uptake at 1 h pi (13.98% ID/g) than at 24 h pi (11.34% ID/g). In the heart, lungs, spleen, kidneys and blood, the radioactivity significantly decreased at 24 h ($p < 0.05$). The bone/blood ratio was 55.76 and 98.86, but the bone/muscle ratio was 152.32 and 470.50 at 1 h and at 24 h post injection, respectively. Compared with DPD labeled with ^{90}Y at pH 7.5, ^{90}Y–DPD labeled at pH 3.0 displayed a significantly different bone uptake at 1 and 24 h post injection ($p < 0.05$). The radioactivity in the bone was higher at 24 h pi (13.12% ID/g) than at 1 h pi (10.54% ID/g). The bone/blood ratio was 37.05 and 201.85, but the bone/muscle ratio was 502.08 and 1009.23 at 1 and 24 h pi, respectively. No significant activity was found elsewhere.

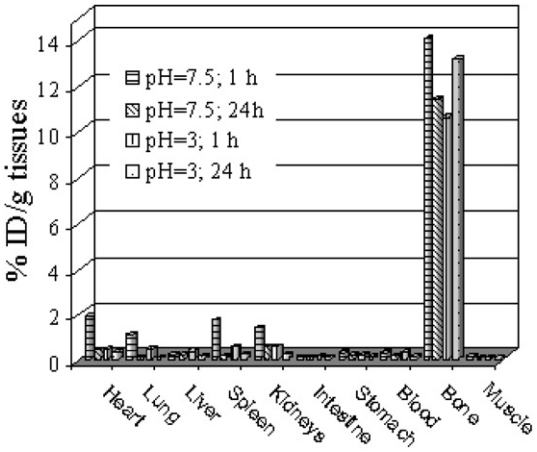


Figure 7. Organ uptake of ^{90}Y–DPD labeled at pH values 3 and 7.5 in the Wistar rats sacrificed at 1 and 24 h pi (n = 3–5; % ID/g).

The uptake of ^{90}Y–DPD in rat is compared with the values obtained for ^{90}Y–(Sn)–DPD complexes and commercial ^{99m}Tc–DPD and is presented in Figure 8. All complexes were prepared under similar pH conditions (pH 7.5). At 1 h after administration of radioactive complexes, ^{90}Y–DPD showed significantly increased bone uptake ($p < 0.05$) in comparison with ^{99m}Tc–DPD. The bone/blood ratio was 42.10 and 55.76, while the bone/muscle ratio was 168.42 and 152.32 for ^{99m}Tc–DPD and ^{90}Y–DPD, respectively. Compared to ^{99m}Tc–DPD complex, the complex ^{90}Y–(Sn)–DPD showed slightly lower the bone (7.56%), lung, spleen and kidneys uptake, while slightly higher the liver and muscle uptake. The bone/blood ratio was 20.44 and the bone/muscle ratio was 103.3.

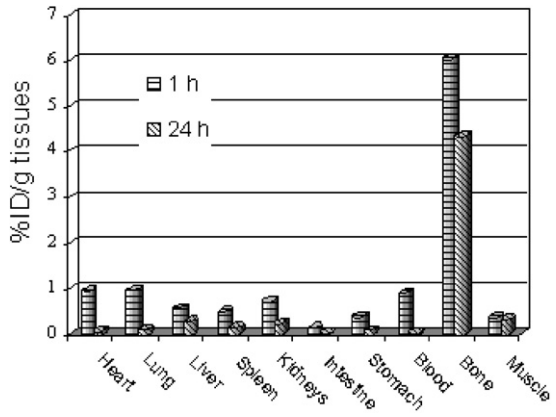


Figure 6. Biological behavior of $^{90}YCl_3$, pH 7.5, in the Wistar rats sacrificed at 1 and 24 h pi, values reported are % ID/g, n = 3–5.

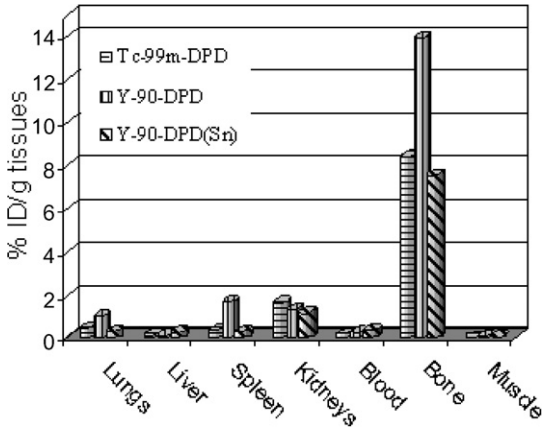
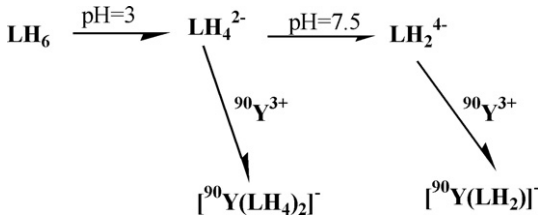


Figure 8. Organ distribution of ^{99m}Tc–DPD, ^{90}Y–DPD, and ^{90}Y–(Sn)–DPD in Wistar rats sacrificed at 1 h pi (pH 7.5, n = 3–5; % ID/g).

2.6. Molecular modeling

Information on the chemical stability can be investigated by looking at the molecular energy data. The simple rule is: lower is the total energy of the complex (molecule), the more stable is the investigated one. Since single crystals could not be grown for Y-90 complexes, it was thought to be desirable to obtain structural information through molecular modeling. The molecular modeling calculations for the ligand DPD along with the proposed complexes $[^{90}Y(LH_4)_2(H_2O)_2]^-$ and $[^{90}Y(LH_2)(H_2O)_2]^-$ have been carried out using HyperChem™ release 6.03 version that allows for rapid structural building, geometry optimization, and molecular display. Full geometry optimization was carried out employing MM+ force field (calculations in vacuo), the Polak–Ribiere algorithm (conjugate gradient) with convergence of 0.0001 kcal/mol and an RMS gradient at 0.001 kcal/Å mol. The dissociation of the DPD as a strong acid (named LH_6 in protonated form) with six protonation sites (four oxygen's on the phosphonic acid moiety and two carboxylic acid groups) is in progression with increased pH value as presented in Scheme 1. According to literature data for similar phosphonates[30] the overall dissociation constants are presented in case of DPD, so different DPD ionic species may be involved in the structure of $[^{90}Y(LH_4)_2(H_2O)_2]^-$ complex.



Scheme 1. Proposed dissociation process of DPD free acid with increasing pH value.

The structure of the free ligand was calculated, starting from standard bond lengths and bond angles, and its geometry was fully optimized by minimizing the energy with respect to geometrical variables without any symmetry constraint. The obtained energy of geometry optimized structure of DPD was 38.627 kcal/mol with gradient 0.09410 kcal/Å mol.

Complex formation between $^{90}Y^{3+}$ of very low concentration (10^{-6} to 10^{-8} M) and DPD (3.5 mM) in great excess depending on the pH value favored complexes with metal to ligand mole ratio 1:2 and 1:1. The energy optimized structures for the proposed complexes of $[^{90}Y(LH_4)_2(H_2O)_2]^-$ formed at lower pH value (mole ratio 1:2) with energy values 78.172, 91.256, and 104.244 kcal/mol, respectively, are presented in Figure 9a–c. The structure of the proposed complex $[^{90}Y(LH_2)(H_2O)_2]^-$ formed at higher pH value (mole ratio 1:1) is presented in Figure 10, the energy of these optimized structure is 59.172 kcal/mol.

3. Discussion

The therapeutic radiopharmaceutical must have the following characteristics: high tumor uptake, high tumor-to-background ratio, long tumor residence time, and fast renal clearance. Coordination chemistry plays a significant role in the development of therapeutic radiopharmaceuticals. The most common way to increase the thermodynamic stability and kinetic inertness of a metal complex is to use a polydentate chelator, which

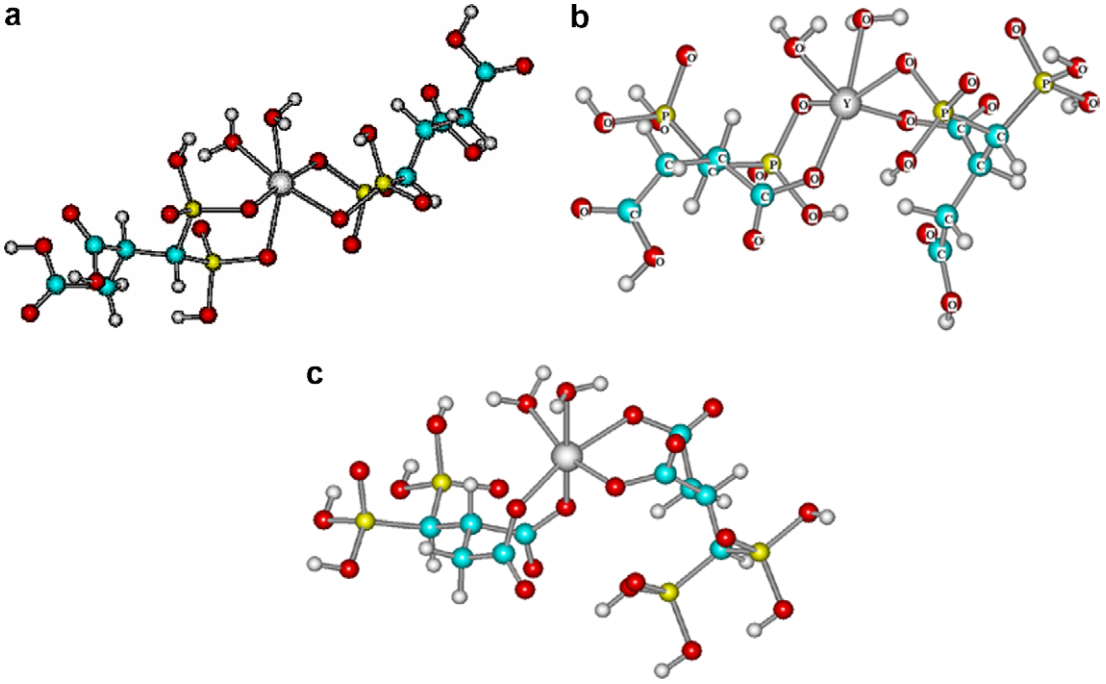


Figure 9. The energy minimized structures of proposed complexes $[^{90}Y(LH_4)_2(H_2O)_2]^-$.

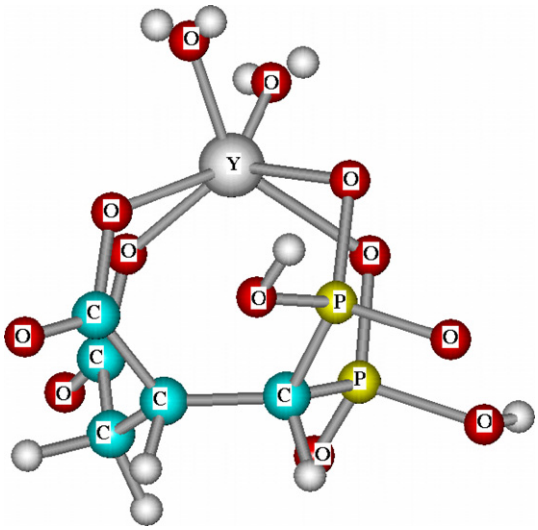


Figure 10. The energy minimized structure of proposed complex $[^{90}Y(LH_2)\ (H_2O)_2]^-$.

should have high hydrophilicity to improve blood clearance and renal excretion of the labeled complex. The 2,3-dicarboxypropane-1,1-diphosphonic acid (DPD) as tetradentate ligand has good chelation properties.

In the present study, in order to achieve the optimal conditions for labeling DPD with ^{90}Y in the form of $^{90}YCl_3$ several factors must be considered. The concentration of DPD substance influenced on the labeling yield. In this study, the highest labeling yield (~98%) resulted with a ligand concentration higher than 0.1 mg/ml (Fig. 2). The heating step during labeling was essential to get a product with high labeling yield and improved stability. The experimental results suggest that quantitative labeling could be achieved with heating at a reaction temperature of 95 °C. A minimum of 30 min heating in water bath for completion of reaction was required (Fig. 3).

It seems that special conditions of pH values were not necessary for quantitative labeling of DPD with ^{90}Y. During the study of the effect of pH value on the labeling yield, by adjusting the reaction mixture to different pH values before adding ^{90}Y, it was observed that pH value did not have a significant effect on the labeling efficiency (Fig. 4). The stability of the ^{90}Y–DPD complex, prepared at optimized conditions was monitoring up to 24 h. This ^{90}Y–DPD complex was stable up to 10 h, which meets the requirement for treatment. The ^{90}Y–DPD complex prepared at a.t. was found to be unstable within 4 h. The experiment clearly suggests that, the heating step during the labeling is essential to get a product with improved stability (Fig. 5).

The stability of the radiopharmaceutical is a very important parameter, because much of the ^{90}Y released in free form will be taken up by the skeletal system and only be released with a very long metabolic half life. Our stability results are promising. In vitro, mixed with serum, higher than 95% of the radioactivity was still DPD bound after 10 days.

Significantly lower binding of ^{90}Y–DPD to the human albumin in comparison with ^{99m}Tc–DPD was observed. This result is in accordance with literature data for some of the ^{90}Y–MDP complexes.[9] As expected, the ^{90}Y–DPD complex posses a hydrophilic character as well as Tc-99m complex.

If free ^{90}Y is injected in a human subject, about 50% of the injected dose will localize in the bone, 25% of the injected dose will go to the liver, 10% of the injected dose is evenly distributed in many other organs and tissues while only 15% of the injected dose will be excreted via the renal system.[31] In animal model, the tissue distribution results of an aqueous solution of $^{90}YCl_3$ pointed out that radioactivity is also distributed mainly in the bone (6.02% ID/g is about 42% in bone) and than slowly decreased with time. The radioactivity in other organs was cleared within 24 h, but there was a very slow clearing activity from the muscles (Fig. 6).

The tissue distribution data for the ^{90}Y–DPD, prepared at pH 7.5 and 3.0 1 h after intravenous administration, were quite different than the data for the ^{99m}Tc–DPD performed on the same animal models. In our study, the biodistribution data showed that ^{90}Y–DPD is significantly accumulated in the bone tissue and remains there for a long time. The uptake in bone was calculated from the activity observed in the femur. The bone uptake of ^{90}Y–DPD, at pH 7.5, was the highest after the first hour. Thereafter the radiotracer washed out from the bone tissue very slowly. The radioactivity in the muscle and blood was low and declined very quickly. At pH value about 3.0 the ^{90}Y–DPD complex also displays highly selective uptake in the skeletal system. The bone uptake increased from the first hour up to 24 h, while the soft tissues uptake is in negligible quantities. We can conclude that while the influence of pH value on the labeling yield is negligible, complex formation is strongly affected by pH value of the solution. The difference in the structure between complexes formed at different H^+ concentrations is a reason for their different uptake in animals.

The complex ^{90}Y–DPD showed a significantly higher uptake in the bone ($p < 0.05$) compared to ^{90}Y–(Sn)–DPD and ^{99m}Tc–DPD complexes formed at the same conditions (see Fig. 8). A possible explanation for the discrepancy between these data may be related to the structure and stability of the complexes. Very low human albumin binding value of ^{90}Y–DPD complex at pH 7.5 is indicative. Also, we suggest that the influence of Sn^{2+} ions in ^{90}Y–(Sn)–DPD formulation is negative due to the stability decrease of complex between ^{90}Y and DPD substance. Because of that, complex accumulation in the bone is significantly lower compared to the ^{90}Y–DPD complex. The ^{90}Y–(Sn)–DPD showed slightly lower bone uptake than those seen for ^{99m}Tc–DPD ($p > 0.05$) and those results also pointed out the influence of Sn^{2+} ions on the ^{90}Y-complex stability.

The Yttrium ion is in the +3 oxidation state, since *4f* electrons are not involved in bonding; interactions between donor atoms and lanthanide metal ions are

predominately ionic. In aqueous solution yttrium ion is coordinated by a number of water molecules. The metal chelate formation process involves the replacement of water molecules by a chelator.[32]

There is no literature data about protonation/dissociation constants for 2,3-dicarboxypropane-1,1-diphosphonic acid and/or stability constants of its complexes. Also, the structure of DPD complexes is not investigated, so we have to include molecular modeling method to obtain any data about ^{90}Y–DPD complexes. The ligand DPD has been defined as H_6L, but it seems that two phosphonic acid OH groups are present in protonated form under the investigated pH region.[33] Dissociation of DPD is in progress with increasing pH value (Scheme 1), two protons are dissociated at pH 3 and LH_4^{2-} species are predominant in solution. But this ionic form (because of the overlapping constants) probably include three structures of DPD^{2-} and three different complexes with the proposed formula $[^{90}Y(LH_4)_2(H_2O)_2]^-$ are formed in the presence of the $^{90}Y^{3+}$. Molecular energy data for those complex structures suggest that structure with lowest energy value (78.172 kcal/mol) is the most stable one (Fig. 9a). This structure involved metal bonding via both phosphonic groups with two molecules of DPD, while two six-membered chelate rings improved the stability of the proposed complex. The other two structures (Fig. 9b and c) include metal–ligand bonding via –P–OH and –COOH or two –COOH groups, respectively, while seven-membered chelate rings slightly decrease the stability of the proposed complexes.

Further increasing the pH value up to 7.5 favored dissociation in the next two protons from LH_4^{2-} and the resulting LH_2^{4-} species are now predominant in solution. The resulting complex with proposed structure presented in Figure 10, which include metal to ligand mole ratio 1:1 and with the lowest energy of the optimized structure (59.172 kcal/mol) is the most stable one. The energy minimized structures of proposed ^{90}Y–DPD complexes include two coordinated water molecules which increased their stability.

4. Conclusions

These experimental studies clearly show that DPD, as the tested ligand, is capable of forming stable complexes with ^{90}Y. Serum stability studies indicate that the DPD forms a more stable complex with ^{90}Y (III) because there is no measurable loss of ^{90}Y (III) from the complex for up to 10 days. The structure of ^{90}Y–DPD complexes strongly depends on the pH value, which is confirmed via molecular modeling energy data. Those complexes were obtained in good yields and were found to be stable in investigated conditions. Those differences in the structures of ^{90}Y–DPD complexes are a reason for their different biological behavior. All of those complexes need to be characterized which will be the object of further investigations. Investigation has to be made on localization of these ^{90}Y-complexes in tumor-bearing rats. In the healthy rats, obtained complexes appear to possess remarkable biological properties.

5. Experimental

5.1. Materials and methods

5.1.1. Chemicals. The substance 2,3-dicarboxypropane-1,1-diphosphonic acid (DPD), was synthesized in 'Vinča' Institute, Department for radioisotopes, by following reported procedure.[25] The commercially available ('Vinča') inactive freeze–dried lyophilized DPD kit was used for labeling. $^{99m}TcO_4^-$ was obtained from ^{99}Mo/^{99m}Tc generator ('Vinča' Institute) and commercially available ('Vinča'). All other reagents and solvents used in these studies were obtained from commercial sources without further purification. $^{90}YCl_3$ was purchased from Polatom, Poland in a no-carrier-added form (29.64 GBq/cm^3, in 0.05 M HCl).

5.1.2. Instrumentation. The Capintec CRC-15 beta counting calibrator (Ramsey, NJ USA) with a calibration factor fixed at 48×10 was used. The radioactivity of each piece of the organs and of the blood was counted on a NaI(Tl) well-type gamma counter (LKB Wallac Compu Gamma Counter). All measurements were carried out under the same geometric conditions. All samples were made up to a 1 ml volume with water to establish a linear relationship between counting rate and samples size. Raw radioactivity data were corrected for background and tested for quantization limits according to literature method.[34]

For the HPLC analysis a Liquid Chromatograph, Hewlett Packard 1050, S/N with UV and Raytest gamma flow detector, and a Zorbax C18 column (250×4.6 mm, 80 Å pore size) was used.

Heating was performed in a temperature controlled heating bath (Sutjeska, Belgrade, Serbia). Molecular modeling of some of the complexes was carried out using HyperChem™ release 6.03 for windows.

All experiments were carried out in triplicate.

5.1.3. Preparation of ^{90}Y complexes

5.1.3.1. ^{90}Y–DPD preparation. The direct labeling method was optimized by varying DPD concentration in the labeling mixture, pH, the reaction temperature and the reaction time. The stock solution of DPD was prepared by dissolution in double distilled water. The desired ligand concentration (0.01–10 mg/ml) was placed in a vial and an appropriate amount of the ^{90}Y–chloride solution was added (~370 MBq per vial). The pH values of the resulting reaction mixtures were adjusted to 3, 5.5 and 7.5 using the different buffers. The sodium acetate buffer (0.1 M) was used to maintain a pH value up to 5.5–6.0; 0.1 and 0.5 M ammonium acetate buffer for pH 7.0–7.5.

The total reaction volume in each vial was maintained at 3 ml. Ascorbic acid (10 mg) was used as radiolytic stabi-

lizer in all samples. Reagent concentrations and time of the reaction were then optimized under the desired pH. In order to optimize the reaction conditions, radiolabeling was performed using a constant amount of the radionuclide and increasing amounts of ligand. The effect of heating and time required for reaction was also studied. The reaction mixtures were kept at room temperature (a.t.) for 0.5–24 h, or were placed in a water bath at 50 and 95 °C for 15, 30, and 60 min. After cooling down to a.t., the complex yields and the radiochemical purity were determined.

The radiochemical purity of the radiolabeled DPD was studied by use of paper, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in all the cases.

Chromatography: The labeling yield of the ^{90}Y-labeled samples was determined using ascending paper and thin-layer chromatography:

1. Paper chromatography on Whatman No. 1 with 0.1 M phosphate buffer pH 6.5 as the mobile phase (free ^{90}Y at $R_f = 0.0–0.1$; ^{90}Y-complex at $R_f = 0.7–1.0$);
2. Paper chromatography on Whatman No. 1 with pyridine–acetic acid–water (5:3:1.5, V/V) as the mobile phase (free ^{90}Y at $R_f = 0.0–0.1$; ^{90}Y-complex at $R_f = 0.8–1.0$);
3. TLC on silica gel 60 strip using ethyl acetate–ethanol (1:1, V/V) as the mobile phase (free ^{90}Y at $R_f = 0.9–1.0$; ^{90}Y-complex at $R_f = 0.0–0.2$).

The 5 μl of the reaction mixture was spotted at 2 cm from the bottom of the strip (2.5 × 20 cm) and developed in a mobile phase until the solvent reached the top of the strip. The strips were dried and cut into 1-cm sections and the radioactivity was measured with NaI(Tl) detector to determine the yield of the radiolabeling and thus radiochemical purity of the complex. Radiochemical purity (RCP) is calculated by considering the peak areas. The activity corresponding to the ^{90}Y-complex peak compared to the total activity on the strip as 100% provides the radiochemical purity.

HPLC analysis: High-performance liquid chromatography method is applied used a Liquid Chromatograph, the flow rate of mobile phase was 0.75 ml/min. and UV-detector is set at 215 nm. The elation was isocratic with mobile phase as followed: 85% solvent A (0.05 mol dm^{-3} phosphate buffer, pH 4.5) and 15% solvent B (acetonitrile).

The stability of the prepared ^{90}Y–DPD complex, stored at a.t., was studied at different intervals of time at the pH optimized for labeling.

5.1.3.2. ^{90}Y–(Sn)–DPD preparation. Radiolabeling of the lyophilized DPD kit with ^{90}Y is performed in a similar manner: the lyophilized DPD kit was reconstituted by adding $^{90}YCl_3$ solution (~370 MBq per vial), the pH value of the reaction mixture was adjusted to 7.5 using the phosphate buffer and vial is placed into a water bath at 95 °C for 30 min. Each vial of the inactive freeze–dried DPD kit contain 11.0 mg 2,3-dicarboxypropane-1,1-diphosphonic acid, 0.5 mg $SnCl_2 \times 2H_2O$, 2.0 mg (4-aminobenzoyl)-glutamic acid and 40 mg NaCl in inert atmosphere. The pH value of preparation was around 7.5. The total volume was 10 ml.

5.1.4. Preparation of ^{99m}Tc complex. To prepare ^{99m}Tc–DPD, the freshly eluted $^{99m}TcO_4^-$ solution, obtained by elution of ^{99}Mo/^{99m}Tc-generator (eluted previously at 24 h), was added to a lyophilized DPD kit according to the instructions of the manufacturer. The lyophilized product was reconstituted by adding 1.0–1.5 mCi (37–55.5 MBq) of Tc-99m pertechnetate in 10 ml of physiological saline. Instant thin-layer chromatography using silica gel (ITLC-SG) and saline as mobile phase was used to detect colloidal or reduced and hydrolyzed Tc-99m, while paper chromatography (Whatman No. 1) and acetone as mobile phase were used to detect free pertechnetate.

5.1.5. Serum stability studies. DPD was labeled with ^{90}Y and its stability in human serum was evaluated up to 10 days. Human serum was prepared by allowing blood collected from a healthy volunteer to clot 1 h at room temperature in a closed tube. The sample was centrifuged and the supernatant serum was transferred to sterile plastic culture tubes. It was then incubated overnight at 37 °C in a humidified 5% carbon dioxide, 95% air atmosphere. The pH of an aliquot was measured as 7.4 before the addition of ^{90}Y–DPD and maintained at pH 7.4 ± 0.1 throughout the experiment. In triplicate, 100 μl of labeled DPD was then added to 2.0 ml of serum and then returned to incubate at 37 °C in a CO_2-enriched atmosphere (5%CO_2). At each time point (1, 2 and 4 h and 1, 2, 3, 5, 7, 10 d) the percentage of ^{90}Y activity dissociated from the ligand was assessed by paper chromatography (Whatman No. 1) using pyridine–acetic acid–water (5:3:1.5, V/V) as the mobile phase. Serum aliquots (10 μl) were spotted on the chromatography paper and allowed to run 12–14 cm from the origin. ^{90}Y-labeled DPD moved to the solvent-front and soluble free $^{90}YCl_3$ remained at the origin in this condition.

5.1.6. Protein binding and lipophilicity measurements. For determination of the percentage of ^{90}Y-labeled DPD bound to 12% human albumin (HA, National Blood Transfusion Institute, Belgrade) during incubation at 37 °C for different time intervals, trichloracetic acid (TCA) precipitation method was used.[25,35] All samples for determination of the lipophilicity were prepared by solvent extraction method with *n*-octanol equilibrated with 0.15 M phosphate buffers (pH 3.5–7.5).[26,36] The measurements were performed at room temperature.

5.1.7. Animal studies. Biodistribution studies were performed in male healthy Wistar rats weighing between 100 and 120 g (4 weeks of age, three to five animals per experiment). The animals were injected with 0.1 ml (18.5–37.0 MBq) of the labeled complex into the tail vein. The animals were sacrificed at 1 and 24 h pi. During 24 h period the rats were kept in special cages having

free access to food and water. Major tissues of the body were removed and assayed for radioactivity in comparison to a standard source. The radioactivity was measured in NaI(Tl) detector and percentages of injected dose in the tissues were calculated. The results are expressed as the percentage of injected activity per organ or gram of tissue (%IA/g). The entire animal study conformed to ethical guidelines and complied with the United Kingdom Biotechnology and Biological Sciences Research Council's Guidelines on the Use of Living Animals in Scientific Investigations. The animals were maintained at a well-ventilated, temperature-controlled (30 ± 1 °C) animal room for few days prior to the experimental period and provided with food and water. The animals were acclimatized to laboratory conditions before the test.

5.1.8. Statistical analysis. All experimental data are expressed as mean ± standard deviation (SD). Statistical significance in all experiments was determined by one-way analysis of variance (ANOVA) and independent *t*-test. p values <0.05 were considered significant.

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References and notes

1. Hoskin, P. J. *Pain* **1995**, *63*, 137.
2. Pandit-Taskar, N.; Batraki, M.; Divgi, R. C. *J. Nucl. Med.* **2004**, *45*, 1358.
3. Casciato, D. A.; Chansky, H. A. In *Manual of Clinical Oncology*; Casiato, D. A., Lowitz, B. B., Eds., 4th ed.; Lippincott Williams Wilkins: Philadelphia, 2000; pp 601–614.
4. Arano, Y.; Ono, M.; Wakisaka, K.; Uezono, T.; Konishi, J.; Yokoyama, A. *Radioisotopes* **1994**, *44*, 514.
5. Collins, C.; Eary, J. F. C.; Donaldson, G.; Vernon, C.; Bush, N. E.; Petersdorf, S.; Livingston, R. B.; Gordon, E. E.; Chapman, C. R.; Applebaum, F. R. *J. Nucl. Med.* **1993**, *34*, 1839.
6. Subramanian, G.; McAfee, J. G. U.S. Patent 4,017,595, 1987.
7. Kozak, R. W.; Raubitschek, A.; Mirzadeh, S.; Brechbiel, M. W.; Junghans, R. P.; Gansow, O. A.; Waldmann, T. A. *Cancer Res.* **1989**, *49*, 2639.
8. Kukis, D. L.; DeNardo, S. J.; DeNardo, G. L.; O'Donnell, R. T.; Meares, C. F. *J. Nucl. Med.* **1998**, *39*, 2105.
9. Malja, S.; Schomacher, K.; Damani, G.; Cuci, T.; Malja, E.; Salaku, A. *Proceedings of an International Sdeminar Held in Hyderabad,* India, January 18–22, 1999; (IAEA-TECDOC-1228), pp 69–78.
10. Volkert, W. A.; Goeckeler, W. F.; Ehrhardt, G. J.; Ketring, A. R. *J. Nucl. Med.* **1991**, *32*, 174.
11. Chinol, M.; Hnatowich, D. J. *J. Nucl. Med.* **1987**, *28*, 1465.
12. Schubiger, P. A.; Hasler, P. H. *Radionuclides for Therapy*; Hoffman-LaRoche & Co. Ltd: Basel, Switzerland, 1986.
13. Deshpande, S. V.; DeNardo, S. J.; Kukis, D. L.; Moi, M. K.; McCall, M. J.; DeNardo, G. L.; Meares, C. F. *J. Nucl. Med.* **1990**, *31*, 473.
14. Meredith, R. F. et al. *Oncology* **1997**, *11*, 979.
15. Pauwels, E. K.; Blom, J.; Camps, J. A.; Hermans, J.; Rijke, A. M. *Eur. J. Nucl. Med.* **1983**, *8*, 118.
16. Vorne, M.; Vahatalo, S.; Lantto, T. *Eur. J. Nucl. Med.* **1983**, *8*, 395–397.
17. de Klerk, J. M. H.; van Dijk, A.; van Rijk, P. P.; Zonnenberg, B. A.; van het Schip, A. D. *J. Nucl. Med.* **1991**, *32*, 1082.
18. Maxon, H. R., III.; Deutsch, E. A.; Thomas, S. R.; Libson, K.; Lukes, S. J.; Williams, C. C.; Ali, S. *Radiology* **1988**, *166*, 501.
19. Body, J. J.; Bartl, R.; Burckhardt, P.; Delmas, P. D.; Diel, I. J.; Fleisch, H., et al. *J. Clin. Oncol.* **1998**, *16*, 3890.
20. Quirijnen, J. M.; Han, S. H.; Zonnenberg, B. A.; de Klerk, J. M.; van het Schip, A. D.; van Dijk, A., et al. *J. Nucl. Med.* **1996**, *37*, 1511.
21. Silberstein, E. B. *J. Nucl. Med.* **1994**, *35*, 1194.
22. Bai, H. S.; Jin, X. H.; Wang, F.; Du, J.; Liu, Y. M.; Chen, D. M. *J. Rad. Anal. Nucl. Chem. Art.* **1996**, *206*, 43.
23. Lipepe, K.; Kropp, J.; Knapp, F. J. R. *Eur. J. Nucl. Med.* **1998**, *25*, 861.
24. Jowesey, J.; Rowland, R. E.; Marshall, J. H. *Radiat. Res.* **1958**, *8*, 490.
25. Vanlić-Razumenić, N.; Vukićević, N. *J. Serb. Chem. Soc.* **1986**, *51*, 63.
26. Brubaker, G. R.; Johnson, D. W. *Coord. Chem. Rev.* **1984**, *53*, 1.
27. HyperChem. Computational Chemistry; Hypercube Inc.; 1996.
28. HyperChem—Getting started, molecular visualization, and simulation. Hypercube Inc.; 1994.
29. Straszko, J.; Paprota, S. In *Molecular Modeling*, Szczecin, Ed.; Technical University, Department of Chemical Engineering and Physical Chemistry, 1998.
30. Mateescu, C.; Princz, E.; Bouet, G.; Mustayeen, A. K. *Phosphorus, Sulfur, Silicon* **2006**, *181*, 947.
31. Shuang, L. *Chem. Soc. Rev.* **2004**, *33*, 445.
32. Liu, S.; Edwards, D. S. *Bioconjug. Chem.* **2001**, *12*, 7.
33. Hamada, Y. Z.; Harris, W. R. *Inorg. Chim. Acta* **2006**, *359*, 1135.
34. Siegel, A. J.; Zimmerman, E. B.; Kodimer, K.; Dell, A. M.; Simon, E. W. *J. Nucl. Med.* **2004**, *45*, 450.
35. Klingensmith, C. W.; Fritzberger, R. A.; Spitzer, M. V. *J. Nucl. Med.* **1984**, *25*, 42.
36. Zoghbi, S. S.; Baldwin, R. M.; Seibyl, J.; Charney, D. S.; Innis, R. B. *Abstracts and Programme*, 12th Intern. Symp. On Radiopharmaceutical, **1997**; Uppsala, Sweden, pp 136–138.