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# In vitro study: binding of $^{99m}$Tc-DPD to synthetic amyloid fibrils

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**ABSTRACT**

This paper is an report of the investigation of the in vitro binding of $^{99m}$Tc-DPD for synthetic amyloid fibrils used for the diagnosis of cardiac amyloidosis (CA), as compared with the use of $^{99m}$Tc-HMDP and 99mTc-PPI. It also includes an inquiry into the role played by $Ca^{2+}$ ions and serum proteins on binding to amyloid like materials, as well as the saturability and specificity of DPD for fibrils versus amorphous precipitates (AP).

In the work, synthetic insulin fibrils (SIF) and AP were characterized by Congo red staining and TEM imaging. An equal amount of three radiopharmaceuticals were then added to fibrils in $Ca^{2+}$ (0-4.2 mmol/L) or human serum (HS) adjoined samples and radiopharmaceutical uptake was assessed. To test the saturability of amyloid binding sites, a displacement assays with cold DPD was performed, while adding 50-1500 nmol of $^{99m}$Tc-DPD to SIF or AP, saturation binding tests were subsequently carried out for evaluating its specificity for amyloid.

Herein, synthetic fibrils and AP showed conformational differences at TEM and polarized microscopy analysis. In our study, $^{99m}$Tc-DPD fibrils uptake was seen to be the highest and increased with calcium ions concentration. What is more, serum proteins reduced the bound fraction to the amyloid deposits of about 15%, and the Kd values of 90 nM and 114 nM relative to SIF and AP, respectively, did not significantly differ.

We saw that $^{99m}$Tc-DPD is the best seeker for amyloid fibrils in cardiac amyloidosis, and that $Ca^{2+}$ concentration positively influenced DPD fibrils binding. Furthermore, the radioactivity bound to the serum protein clear up the idea of nuclide exchanging dynamic balance between amyloid and circulating proteins. Moreover, non-labeled DPD did not exert a competition for $^{99m}$Tc-DPD binding sites, and, finally, DPD cannot be defined a radiopharmaceutical specific for amyloid deposits.

## INTRODUCTION

Amyloidosis are a group of diseases characterized by deposition of different proteins, which form insoluble fibrils and infiltrate various tissues, leading to organ disfunction [22]. These diseases may be systemic or localized and are currently classified according to the type of precursor protein [30]. The three most clinically challenging types of amyloidosis are the monoclonal immunoglobulin light chain amyloidosis (AL), the hereditary mutated transthyretin related form (ATTRm) and wild-type transthyretin related amyloidosis (ATTRwt), also known as Systemic Senile Amyloidosis (SSA). All are different in terms of disease profile and long term outcome and therapy. AL is associated with worse-case prognosis due to more rapid heart failure progression than TTR related diseases. Indeed, heart involvement confers the worst prognosis, so it is accepted that an early cardiac amyloidosis (CA) diagnosis, prior to organ damage, including a correct and in-equivocal amyloid typing, is crucial for an appropriate patient clinical management [18]. To this day, since Kula [20] visualized calcifications in amyloid deposits with $^{99m}$Tc-diphosphonate, nuclear imaging of CA is predominantly accomplished with bone seeking radiotracers: 99m-technetium-labeled

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pyrophosphate ($^{99m}$Tc-PPi) [4,7,10,12], 99m-technetium methylene diphosphonate ($^{99m}$Tc-MDP) [1], and 99m-technetium 3,3-diphosphono-1,2-propanodicarboxylic acid ($^{99m}$Tc-DPD). However, conflicting results in terms of diagnostic performance do occur, since extra-osseous bone uptake of bone tracers has been attributed to a variety of pathological conditions – from myocardial infarction to amyloidosis or neoplastic diseases. The administration of $^{99m}$Tc-DPD seems to help in differentiating AL- and ATTR- type CA [26], suggesting a role for scintigraphy in the evaluation of CA etiology, thus avoiding the need for endomyocardial biopsy [24] allowing to quantify cardiac amyloid load and monitor treatment response. Nevertheless, the exact molecular structure of the amyloid fibrils is not completely understood, as well, the mechanisms of interaction between diphosphonates and amyloid fibrils remain unknown. These observations led us to try to understand the chemistry of $^{99m}$Tc-diphosphonates radiopharmaceutical, their interaction with amyloid deposits and to investigate the role of extracellular components in the $^{99m}$Tc-*bone seekers* (DPD, HMDP and PPi) amyloid fibrils binding. The aim of our work was to investigate the in vitro binding affinity of DPD for synthetic insulin amyloid fibrils, in a comparison with HMDP and Ppi, and to ascertain the role of $Ca^{2+}$ ions and serum proteins on this binding. In addition, we assessed the specific uptake of radiotracers by fibrillar versus amorphous deposit.

## MATERIALS AND METHODS

***Preparation of radiopharmaceuticals***. The commercially available DPD (Teceos – Iba Cisbio), HMDP (Osteocis – Iba Cisbio) and PPi (Angiocis – Iba Cisbio) cold lyophilized kits were used for labeling with freshly eluted $^{99m}TcO4^-$ obtained by a $^{99}$Mo/$^{99m}$Tc generator (Tekcis – Iba Cisbio). $^{99m}$Tc-HMDP, $^{99m}$Tc-DPD preparation (11000 MBq of $^{99m}$Tc pertechnetate in 5 mL of saline) and quality control were performed according to the instructions of the manufacturers. $^{99m}$Tc-PPi (Angiocis – Iba Cisbio) was in vitro reconstituted with 11800 MBq of $^{99m}$Tc pertechnetate in 5 mL of saline and after 30 minutes at 25°C.

***Characterization of $^{99m}$Tc-DPD***. An Agilent 1220 series high pressure liquid chromatography (HPLC), UV-VIS diode array with Zorbax Eclipse XDB C18 column was used for diphosphonates chemical characterization. Isopropanol was utilized as solvent A, $1\times10^{-3}$ M sodium acetate, $2\times10^{-3}$ M tetrabutilammonium hydroxide and 3% v/v ethyl acetate (pH 6 with acetic acid) were employed as solvent B.

***Preparation of synthetic amyloid materials***. Human Recombinant Insulin (10 mg, Sigma Aldrich), was dissolved in 1 mL aqueous HCl solution (pH = 2) and incubated at 60°C for three days, brought about the formation of "amyloid like fibrils". Incubation of the same in aqueous HCl solution (pH = 2) at 60°C, containing 40% (w/w) ethanol (EtOH), resulted in the formation of amorphous aggregates [14]

***Congo red staining and TEM analysis***. The staining solution was prepared as indicated in diagnosis of systemic amyloidosis guidelines [31]; polarized optical microscopy (Nikon Eclipse E 600) and Transmission Electron Microscopy (TEM JEOL JEM 1011 with MORADA Olympus camera) were used for fibrils and amorphous precipitates characterization.

***Radiopharmaceuticals binding assay***. Amyloid material was filtered with polycarbonate Nuclepore 25 mm diameter 0.4 μm pores membranes with plastic swin lok filter holder. A Capintec CRC-15PET radioisotope dose calibrator (Capintec INC.) and a 3 × 3" NaI(Tl) gamma well counter (Raytest) were used for quantitative gamma spectrometry. All measurements were carried out under the same counting geometry, data were corrected for background and decay. For radiopharmaceuticals binding assay, equimolar amounts of three radiopharmaceuticals (140 nmol) in separate experiments were added to 500 μg of synthetic insulin amyloid fibrils and water up to a 1500 μL total volume, in various concentrations of $Ca^{2+}$ (0, 0.7, 1.4, 2.8, 4.2 mmol/L) or in the presence of human serum (HS, 20 μL) collected from a healthy volunteer. After 2 h at 25°C incubation, each sample was spun at 280 g in a Heraeus Labofuge centrifuge to separate supernatants from pellets and then washed with deionized water. Radiopharmaceuticals uptake for each sample was assessed.

***$^{99m}$Tc-DPD saturation binding test***. To test saturability of SIF binding sites, cold DPD molecules (100 μL, 684 nmol) were added to a constant amount of amyloid fibrils (1 mg) in saline containing $Ca^{2+}$ in a concentration of 1.4 mmol/L (final volume 1 mL). After 2 hours incubation at 25°C, in the presence or absence of "cold ligand" excess, $^{99m}$Tc-DPD was added (100 μL, 684 nmol). All samples were incubated for a further two hours and treated as above.

***Influence of serum protein on $^{99m}$Tc-DPD binding***. To establish the influence of serum protein in $^{99m}$Tc-DPD uptake by SIF, 100 μL (684 nmol) of radiopharmaceutical were incubated for 2 hours at 25°C with the same volume of HS. The HS protein component was isolated by TCA 50 % w/v precipitation, spun at 280 g, washed with acetone and counted in a NaI(Tl) scintillator.

***Specificity of $^{99m}$Tc-DPD for SIF***. To assess the $^{99m}$Tc-DPD amyloid specificity and to calculate the dissociation constant ($K_d$), isothermal binding tests with both SIF or AP as substrate were carried out. Ten samples containing 1 mg of SIF or AP and 1.4 mmol/L of $Ca^{2+}$ were added with increasing amounts of $^{99m}$Tc-DPD (from 50 to 1500 nmol, 1 mL total volume) at 25°C and treated as previously described. The specific binding was assessed, $K_d$ (nM [Tc-DPD]) and $B_{max}$ (nmol Tc-DPD/mg substrate) values were calculated according to the "One site – specific binding with (*h*) Hill slope" model (Graph Pad Prism 5.00 software, San Diego, California – USA).

Calcium Gluconate 100 mg/mL solution was purchased from Bioindustria L.I.M. Ultrapure water was produced by Milli-Q System (Millipore). Human serum was collected from a healthy volunteer.

Results are reported as "number" (standard deviation, SD) with a statistically significant p value < 0.05.

## RESULTS

***Characterization of SIF and AP.*** The lab-made synthetic fibrils demonstrated the typical apple-green birefringence with polarized light after Congo red staining, while AP did

not (Figure 1). In addition, TEM images clearly showed conformational differences between fibrils and amorphous material (Figure 2). Moreover, SIF appeared as dense-cored, twisted net of elongated well-defined tubular shape structures, while AP looked like bigger clusters, non-fibrillar ball-like aggregates.

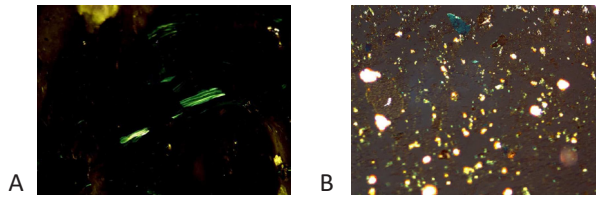


***Figure 1.*** Optical microscopy images of SIF and AP. Images of insulin synthetic fibrils (A) and amorphous precipitates (B) at polarized optical microscopy after Congo red staining

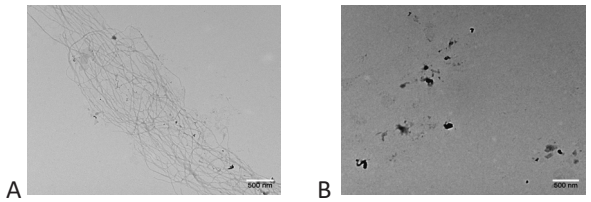


***Figure 2.*** TEM images of SIF and AP. Images of insulin synthetic fibrils (A) and amorphous precipitates (B) gained through Transmission Electron Microscopy (TEM)

***Radiopharmaceuticals binding assay.*** The radiochemical purity of the three radiopharmaceuticals were > 97%, and the pH values complied with the range indicated by the manufacturers. The comparison between three radiopharmaceuticals showed that $^{99m}$Tc-DPD activity bound to fibrils was higher than that of $^{99m}$Tc-HMDP and $^{99m}$Tc-PPi (Figure 3); HS seemed to reduce the radioactivity percentage bound to the amyloid fibrils, namely generating a reduction of 15% (SD = 3%, $p < 0.05$). In addition, $^{99m}$Tc-DPD binding to fibrils seemed to increase with $Ca^{2+}$ ions concentration – from 0 to 2.8 mmol/L.

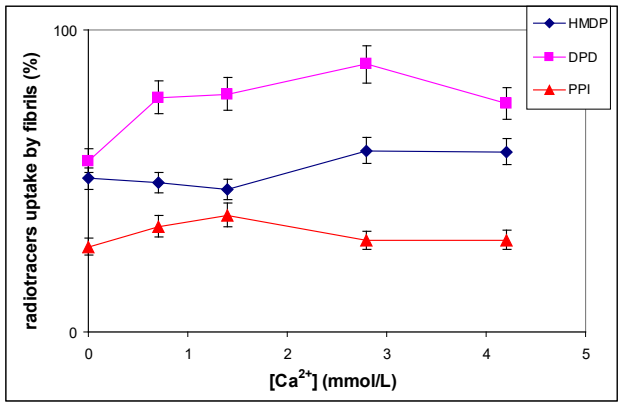


***Figure 3.*** Calcium related $^{99m}$Tc-bone seekers uptake by SIF. Fibrils uptake of three radiotracers at various $Ca^{2+}$ concentration. The uptake of three radiotracers is expressed as a percentage of the total added radioactivity

***$^{99m}$Tc-DPD saturation binding test.*** The saturation binding test revealed that the radioactivity bound to SIF was slightly different (2%, $p < 0.05$) in each sample, regardless of the presence in solution of an excess of DPD as competitor for the binding.

***Influence of serum protein on $^{99m}$Tc-DPD binding***. In the evaluation of role play by this protein in DPD-SIF binding, HS addition decreases the binding to fibrils in terms of radioactivity of approximately 5% (SD = 2%, $p < 0.05$).

***Specificity of $^{99m}$Tc-DPD for SIF.*** The $^{99m}$Tc-DPD specificity for amyloid material is well represented by the comparison of two, fibrillary or amorphous, uptake isotherms (Figure 4). The two sigmoidal curves fitting the data series presented both a good $R^2$ value, so it was possible to calculate $K_d$ and $B_{max}$. The confidence intervals in Table 1, indicated that the $K_d$ values did not significantly differ among the different substrates, instead $B_{max}$ relative to insulinic amorphous material was lower than SIF, probably due to the smaller AP contact surface, so less molecules of $^{99m}$Tc-DPD are necessary to bring about the binding saturation. Data analysis with the "One site – specific binding with (*h*) Hill slope" model revealed *h* values higher than 1 of both sigmoidal curves, hence attesting to the presence of several molecular species labelled in the equilibrium of binding; in fact, these values of Hill coefficient described a sort of cooperativity of ligand binding. The curves showed a similar pattern even if the uptake by fibrillar material is higher at any point.

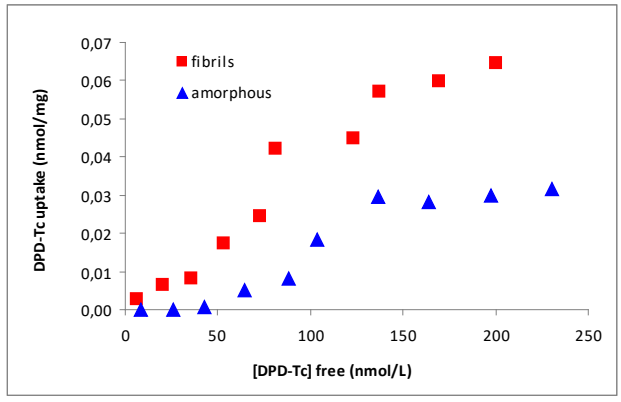


***Figure 4.*** $^{99m}$Tc-DPD uptake by SIF and AP

Synthetic amyloid fibrils (SIF) and proteinaceous amorphous material (AP) $^{99m}$Tc-DPD uptake isotherms at 25°C.

***Table 1.*** Comparison of fibrils versus amorphous precipitate in isothermal uptake

Parameters of "One site – specific binding with Hill slope" model for the two isothermal uptake of Figure 4.

| | FIBRILS | AMORPHOUS PRECIPITATES |
|---|---|---|
| ***Best-fit values*** | | |
| $B_{max}$ (nmol/mg) | 0.07594 | 0.03174 |
| *h* | 2.116 | 4.801 |
| $K_d$ (nM) | 90.04 | 114.3 |
| ***95% Confidence Intervals*** | | |
| $B_{max}$ (nmol/mg) | 0.04319 to 0.1087 | 0.02863 to 0.03484 |
| *h* | 0.754 to 3.478 | 2.977 to 6.625 |
| $K_d$ (nM) | 42.73 to 137.4 | 104.2 to 124.3 |
| ***Goodness of Fit*** | | |
| $R^2$ | 0.9694 | 0.9912 |

## DISCUSSION

Various types of amyloidosis present different clinical profiles and outcomes [6,23], so early diagnosis and typing is crucial for the better management of the disease. Thus, we focused on the ability of bone seekers radiopharmaceuticals to bind amyloid fibrils and distinguish the different cardiac amyloidosis types. Furthermore, there is little information in current literature about chemical-physical characterization of these bone seekers that would be useful to better understand their distribution pattern.

Among the three bone seekers in clinical use, $^{99m}$Tc-DPD is considered useful for discriminating AL from TTR CA [27,28], and its uptake in the clinical setting is higher than $^{99m}$Tc-HMDP [29].

Our results indicate a higher affinity of $^{99m}$Tc-DPD versus $^{99m}$Tc-HMDP and $^{99m}$Tc-PPi. Even though in several cases, $^{99m}$Tc-PPi has shown higher sensitivity than $^{99m}$Tc-MDP [3,15], in our in vitro test, the PPi molecule showed the lesser affinity for SIF, so the current heart $^{99m}$Tc-PPi technique does not appear to be sufficiently sensitive and accurate in early stage assessment of disease. Jurisson [17] previously described MDP as an oligomeric and polymeric mixture where Tc is linked to two diphosphonates, in a sort of linear polymeric chain, and our HPLC results (Figure 5) support the hypothesis that $^{99m}$Tc-diphosphonate radiopharmaceuticals are a complicated mixture of oligomeric and polymeric technetium complexes [21] as result of ligand excess in kit reconstitution [32]. An important issue to consider is that in our binding test, we assumed that the only chemical specie was [Tc(DPD)$_2$], but our samples contained a large excess of free cold DPD besides the various oligomeric chemicals species formed from the Tc$^{n+}$-diphosphonates interaction. Thus, our discussion can only be a simplified explanation of an intricate process. As attested by the data analysis, h values > 1 might be the evidence of the presence of several molecular species with different mechanisms of cooperativity in the ligand-substrate complex formation. Our hypothesis is that technetium atoms could be linked to different DPD molecules, that is, a polymeric ligand formed by many DPD molecules carried several atoms of $^{99m}$Tc.

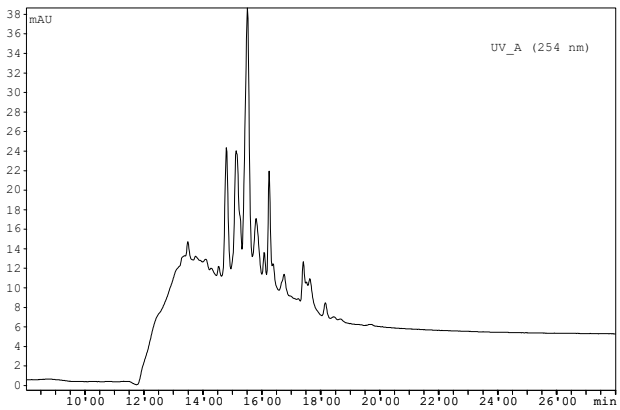


***Figure 5.*** HPLC chromatogram of Teceos. HPLC chromatogram of Teceos (detector UV 254 nm)

Of note: the amyloid fibril structure is not yet exactly known. There is a general agreement that a core protein is $Ca^{2+}$ dependently bound to amyloid P-component (SAP), a plasma protein that may have a stabilizing role, and that glycosaminoglycans (GAG) or proteoglycans are structural components. Fibrils monomers are also known to be orientated perpendicularly to the fibril axis, bound to each other by hydrogen bonds to a very stable β-sheet secondary conformation, and several filaments are twisted around each other to form the amyloid fibril [16].

There is evidence from in vitro analyses that TTR binds to calcium. In addition, its content in different amyloid type fibrils could influence the affinities of diphosphonates to amyloid proteins, and might explain the heterogeneous results found in previous studies – among these, the mild uptake in patients with AL rather than the strong uptake in individuals afflicted with ATTR [29]. Yood [33] suggests that the high calcium concentration in amyloidotic deposits supports the binding. Moreover, an ionized calcium gradient and acid phosphatase content are among the factors involved in the mechanism of PPi accumulation in pleural effusions [2]. Therefore, in our work, we tried to investigate the role of calcium ions in the radiopharmaceuticals deposit interaction. The result of this study is that $Ca^{2+}$ is probably involved in radiopharmaceutical fibrils binding at higher concentration than it does under normal physiological conditions. Several studies on $^{99m}$Tc-diphosphonate bone agents have compared the biological properties of the clinically used agents [9,11], and it appears that the skeletal uptake of $^{99m}$Tc-diphosphonate bone agents increases exactly in a manner corresponding to the order determined for the calcium affinity. This class of molecules accumulates in all calcium intensive metabolic activity areas, such as the skeleton or recent infracted myocardium, doing so by ionic exchange and bone organic matrix adsorption. This process occurs in the healthy bone, but accumulation is higher at sites characterized by increased bloodstream and osteoblastic activity [5] resulting in an aspecific binding. It is possible that amyloid affected myocardial tissue acts like necrotic myocardial tissue [25] where radiopharmaceuticals localization is the result of irreversible deposition of calcium in the mitochondria forming granular structures similar to crystalline hydroxyapatite. This supports the non-selective binding found in our comparison between SIF and AP.

The presence of HS in the radiopharmaceutical solution leads to a protein radioactivity uptake equal to the 5% of the total activity, which confirms data attesting a serum protein removal from 5 to 10% of total radioactivity [19], together with the non-linear binding trend, defined as "ionic exchange" between albumin and $^{99m}$Tc-diphosphonates complexes. Alternatively, it could be explained by the replication of dynamic equilibrium between free radiopharmaceuticals in blood, available for pharmacodynamic interaction on their own site of action, and the share bound to plasma proteins. In addition, calcium binding proteins such as albumin, decrease the ionized amount, thus reducing uptake [2].

## CONCLUSIONS

The early diagnosis of cardiac involvement, prior to organ damage, and the amyloid typing, lacks a punctual diagnostic criteria in a context where new therapies require an accurate patient stratification and endomyocardial biopsy remains a-not-without-risk procedure. $^{99m}$Tc-DPD is confirmed to be the best tracer for use, in comparison with the other two bone seekers, showing higher affinity than $^{99m}$Tc-HMDP and $^{99m}$Tc-PPi. Nevertheless, clinical value of $^{99m}$Tc-DPD for the assessment of disease severity and prognosis especially in SSA has not been evaluated extensively [13]. The diagnostic possibilities of $^{99m}$Tc-DPD in amyloidosis have not yet been fully assessed, and in our experiments, we observed that the calcium ions content of the media seems to play an important role in the binding process at higher ion concentration than does the physiological (1.4 mmol/L).

The saturation $^{99m}$Tc-DPD binding curves for fibrils or amorphous precipitates showed not significantly different affinity, hence, bring about a situation wherein it is not possible to define a radiopharmaceutical specificity for amyloid fibrils, indicating a situation of non-specificity for amyloid deposits. In addition, cold DPD seems not to exert a relevant influence for $^{99m}$Tc-DPD binding. The radioactivity bound to serum protein clears up the idea about the interaction mechanism being based on the nuclide ionic exchange between fibrillar and circulating protein substrate, and being explainable by a dynamic balance.

The complete comprehension of the whole process is not definitively solved and amyloid imaging techniques remain a promising challenge for further studies in Nuclear Medicine.

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