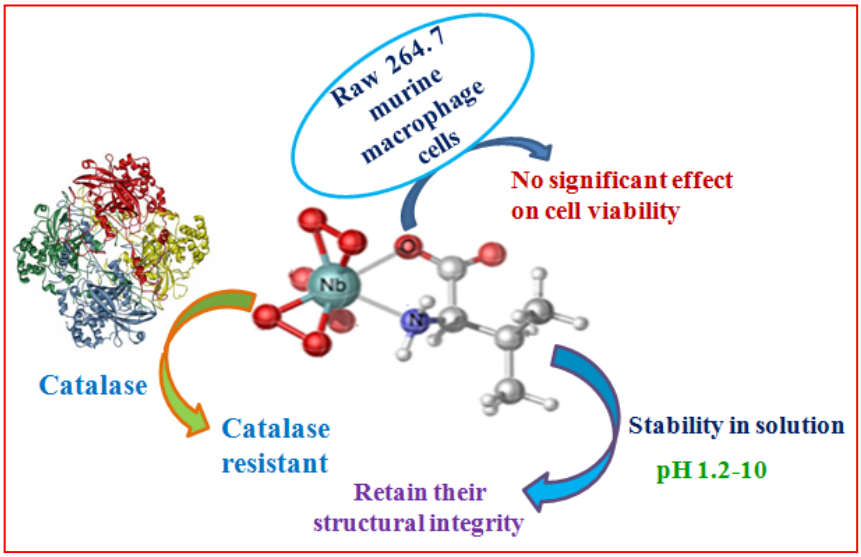
CHAPTER 6

Chapter 6

Studies of Bio-relevant Properties of Peroxoniobium(V) Compounds: Hydrolytic Stability, Cytotoxicity and Their Interaction with Catalase

Contents

It has been demonstrated that the pNb compounds, **NaNb**, **KNb**, **3.1**, **3.2**, **4.1-4.4** retain their structural integrity in solution of pH values ranging from 1.2-10. The compounds also exhibit remarkable resistance to catalase action. The cytotoxicity of pNb compounds towards Raw 264.7 murine macrophage cells was assessed by determining cell viability employing MTT assay. No significant effect on cell viability was observed even at relatively higher compound concentration (200 μM) reflecting their minimal toxicity to the cells.



6.1 Introduction

One of the most important criteria required to be met by water soluble metal complexes to be useful as pharmacological or biorelevant agent is the hydrolytic stability of such species. In this context, it is pertinent to mention that out of the multitude of synthetic pV compounds tested for possible therapeutic activities in recent years, to our knowledge only few compounds have been clinically tested on human [1-5]. One possible factor responsible for limiting the therapeautic potential of pV compounds is their hydrolytic instability and toxicity [5-7]. As a case in point, orally administered pV was found to be ineffective in inducing normoglycemia in STZ-rats by Shisheva *et al.* [8] probably because it could not survive the strong acidity of the stomach. Consequently, the quest for biologically relevant pV compounds spurred by the need for stable, better absorbed, more efficacious vanadium compounds with therapeutic potential still continues. It is therefore somewhat surprising to note that notwithstanding the knowledge that pNb compounds are relatively more stable than pV and Nb possesses several favourable bio-relevant properties including low toxicity, bio-inertness, bio compatibility and chemical stability [9,10], the biochemical potential of peroxoniobium compounds has rarely been investigated [11,12]. Consequently, since one of our major aims of the present study was to examine some of the heretofore unexplored biologically important features of pNb derivatives, such as their interaction with various enzyme functions, we deemed it imperative to ascertain the stability of the newly synthesized pNb compounds towards degradation in aqueous solution under varying pH conditions, as well as their cytotoxicity.

An additional concern was to investigate the interaction of the synthesized pNb complexes with catalase, the ubiquitous enzyme responsible for the decomposition of H_2O_2 to H_2O and O_2 in the intercellular peroxisome [13]. Being a reactive oxygen species (ROS), hydrogen peroxide has long been known as a major contributor to DNA damage, protein oxidation and lipid peroxidation, which can ultimately lead to cell death [14-16]. In recent years however, the view that H_2O_2 is a toxic waste product has changed following the reports documenting its importance as a key signal transducing agent which regulate a variety of cellular processes [17-25]. Hydrogen peroxide is unstable inside the cell as it is effectively degraded and scavenged by cellular catalase and glutathione peroxidase, that has been found to be a hindrance for studying the

signalling activities of H_2O_2 [18-21]. Most experiments on cellular effects of H_2O_2 reported in the literature required the use of concentrations of H_2O_2 >100 μM in the medium, manifold higher than physiologically available, to observe its effects [26]. Thus there have been efforts to find peroxide derivatives that can substitute for H_2O_2, which would be less susceptible to degradation by catalase, yet efficient in their action preferably at far lower concentrations. Previous studies demonstrated that diperoxovanadate compound (DPV) can substitute for H_2O_2, as the peroxo group of DPV is less accessible to degradation by catalase and is active as a substrate in horseradish peroxidase reaction in the presence of catalase at 1/100 concentration compared to H_2O_2 [27].

Apart from pV compounds, peroxo compound of Mo and W, synthesized and reported previously from our laboratory, showed reasonable resistance to catalase action [28-33]. However, no report seems to be available on the interaction of catalase with discrete peroxoniobate compound in solution. Since Nb also belongs to the same group as V, we anticipated that pNb compounds would display similar behaviour toward catalase, creating new perspective for an extended bio-relevant aqueous chemistry of this rarely used element.

Presented in this chapter are the findings of our studies on the stability of the synthesized complexes, homoleptic tetraperoxoniobate **NaNb** and **KNb,** macrocomplexes **3.1**, **3.2** and monomeric pNb complexes **4.1-4.4** towards degradation in solution of a wide range of pH values, as well as the assessment of their interaction with catalase *vis-à-vis* H_2O_2, its natural substrate. Furthermore, results of the investigation on the effect of pNb compounds on the viability of murine macrophage cells are also reported herein. We endeavored to draw comparison between the two types of pNb compounds, macro as well as neat, with respect to their tested properties.

6.2 Experimental section

6.2.1 Stability of the compounds 3.1 and 3.2 as well as 4.1-4.4 in aqueous solution

In order to assess the stability of the compounds in solution, a stock solution (100 mL) was prepared for each of the compounds maintaining the initial compound concentration as 0.2 mM, by adding weighed amount of the respective compounds **NaNb**

(0.105 mg/mL), **KNb** (0.068 mg/mL), **PANb** (**3.1**, 0.096 mg/mL), **PSSNb** (**3.2**, 0.123 mg/mL), **NbAla** (**4.1**, 0.065 mg/mL), **NbVal** (**4.2**, 0.070 mg/mL), **NbA** (**4.3**, 0.089 mg/mL) or **NbN** (**4.4**, 0.079 mg/mL). The peroxide content was then determined in aliquots drawn from the stock solution at different time intervals by the procedure described in Chapter 2, for a period of 12 h. Stability of the compounds in solutions at pH 4.6, pH 7.0 and 8.0, respectively, maintained by using phosphate buffer (50 mM, pH 4.6, pH 7.0 or 8.0), at pH 1.2 and 2.1 (50 mM KCl/HCl buffer), and at pH 3.1 (50 mM citrate buffer) was similarly measured. Moreover, ^{1}H NMR and ^{13}C NMR spectra of the compounds were monitored at 30 min time intervals for any possible change over a period of 12 h, as a measure of stability of the compounds in solution (**Fig. 6.1** and **Fig. 6.2**). For polymer-anchored pNb compounds, the compound concentrations were on the basis of actual peroxoniobium loading (mmol g^{-1}).

6.2.2 Effect of catalase on the pNb complexes

The extent of catalase induced decomposition of the compounds was evaluated by determining the change in peroxide content of the compounds in a solution consisting of the pNb compound in phosphate buffer (50 mM, pH 7.0) and the enzyme, at specified time intervals as has been reported earlier [28-33]. The test solution containing the phosphate buffer (50 mM, pH 7.0) and catalase from bovine liver (40 μg/mL) was incubated at 30 °C. The volume of the test solution was kept at 100 mL. The weighed amount of the respective pNb compound, as mentioned in **Table 6.1**, was then added to the test solution and aliquots of 5 mL were drawn out at intervals of 5 min from the starting of reaction and its peroxide content was determined after quenching the reaction by adding it to cold sulfuric acid (0.7 M, 100 mL). For polymer-anchored pNb compounds, the compound concentrations were on the basis of actual peroxoniobium loading (mmol g^{-1}).

6.3 Results and discussion

6.3.1 Stability of the complexes toward decomposition in solution

The stability of the pNb compounds was examined not only at the natural pH attained by the solution on dissolving the compounds in water, but also under acidic as

well as higher pH conditions of the solutions (**Fig. 6.3**). Compound stability was verified with respect to loss of co-ordinated peroxo groups by determining the peroxide content for any possible change at specified time intervals. From the results it was evident that the peroxide content of the compounds remained practically unchanged even after a period of 12 h. We have further tested and confirmed the stability of each of the pNb compounds in solutions of wide range of pH values such as 1.2, 2.1, 4.6, 7.0 and 8.0, maintained by using appropriate buffer. Furthermore, ^{1}H and ^{13}C NMR spectra of the pNb compounds when monitored over a period of 12 h did not show any significant change (**NbA, Fig. 6.1** and **Fig. 6.2** and for other pNb compounds, **Figs. 6A.1 - 6A.8**). These results are mutually consistent and collectively corroborate that the compounds retain their solid state structure in solution. Thus, this evidence gathered from separate experiments collectively attest to the stability of the compounds in solution.

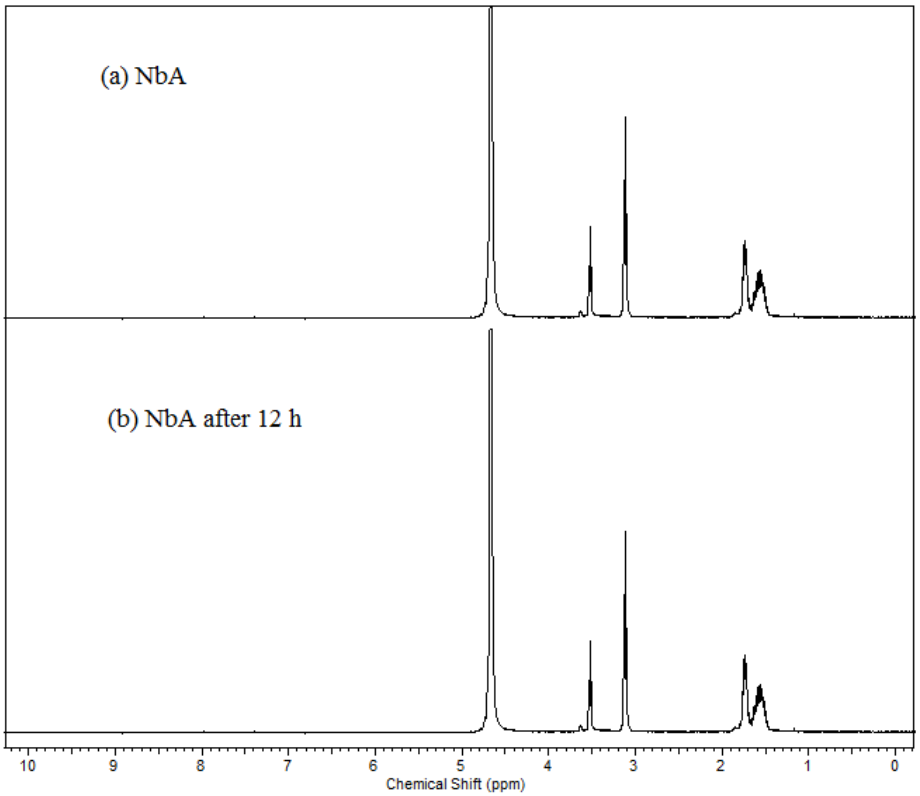


Fig. 6.1 The ^{1}H NMR spectra of **NbA** in D_2O. The spectra were recorded as follows: (a) **NbA** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

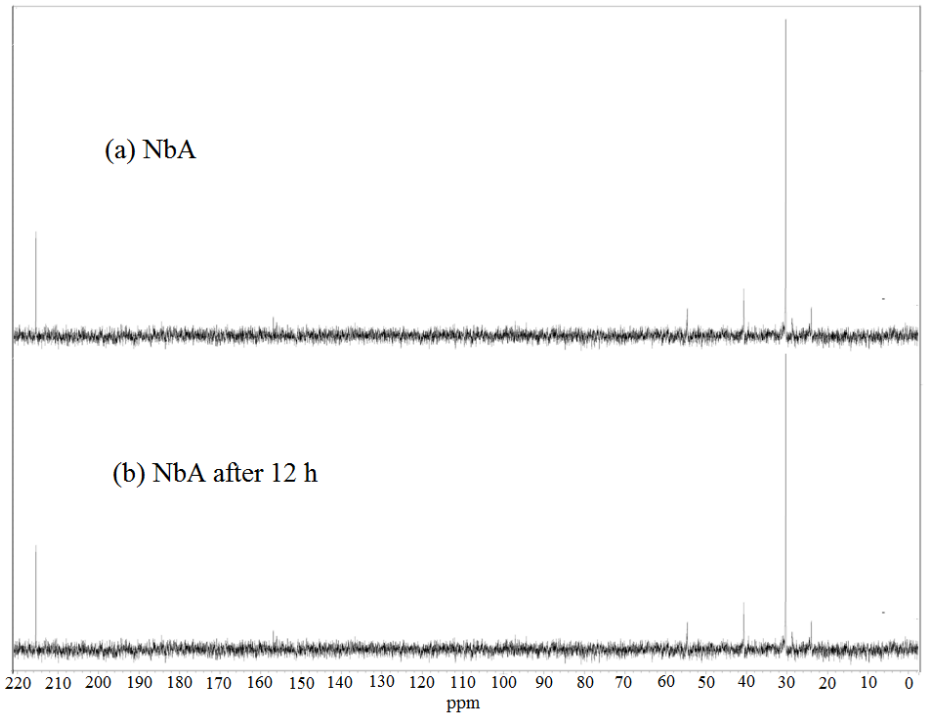


Fig. 6.2 The ^{13}C NMR spectra of **NbA** in D_2O. The spectra were recorded as follows: (a) **NbA** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

6.3.2 Interaction of pNb compounds with catalase

The effect of catalase on the hetero-ligand pNb complexes **3.1**, **3.2** and **4.1-4.4** as well as the tetraperoxoniobate complexes, **NaNb** and **KNb** are shown in **Fig. 6.3**. The data presented in **Table 6.1** demonstrate that in presence of catalase at pH 7, each of the pNb compounds undergoes gradual degradation with loss of peroxide.

On the other hand, it has been reported that addition of catalase to a 0.1 mM solution of its native substrate, H_2O_2 leads to rapid degradation with release of half equivalent of oxygen (molecular basis) with a rate of 430 μM/min as expected from the disproportionation reaction and the process is completed within *ca.* 2 min [34]. This difference in rates of degradation between H_2O_2 and the pNb species indicates that the compounds are at least 20-60 fold weaker as substrate to the enzyme, with respect to H_2O_2. From the above results, it may be surmised that co-ordination of peroxo ligand to Nb enhances its resistance towards degradation under catalase action. It is also notable that the initial rates of degradation of the tested pNb compounds vary from compound to compound (**Table 6.1**) suggesting that the ability of the complexes to withstand catalase

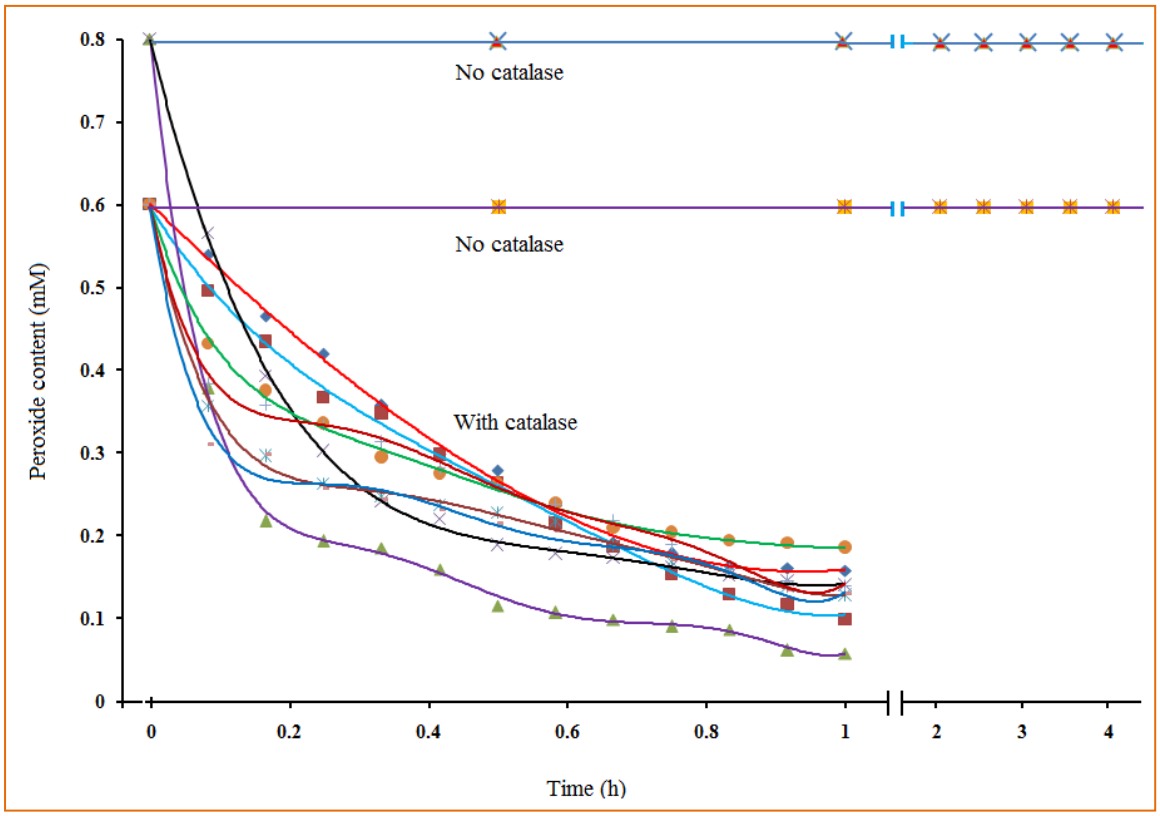


Fig. 6.3 Stability of compound **NaNb** at different pH values: (▲) compound solution in distilled water, pH of the solution = 10.0, (X) solution of complexes in phosphate buffer (50 mM, pH 7.0). Stability of compound **NbA** (**3.3**) at different pH values: (■) compound solution in distilled water, pH of the solution = 9.0, (ж) solution of complexes in phosphate buffer (50 mM, pH 7.0). Effect of catalase on (x) **NaNb**, (▲) **KNb**, (♦) **PANb (3.1)**, (■) **PSSNb (3.2)**, (+) **NbAla (4.1)**, (-) **NbVal (4.2)**, (ж) **NbA (4.3)** and (●) **NbN (4.4)**. The test solution contained phosphate buffer (50 mM, pH 7.0) and the catalase (40 μg/mL) which was incubated at 30 °C for 5 min. Compounds were then added to the reaction solution, aliquots were drawn at indicated time points, and loss in peroxide content was determined. For polymer-anchored pNb compounds, the compound concentrations are on the basis of peroxometal loading.

action is influenced by the auxiliary ligand environment. The pNb compounds could be arranged in the following order of increasing stability toward catalase induced degradation: **KNb < NaNb < NbVal < NbA < NbAla < NbN < PSSNb < PANb**. Relatively greater resistance displayed by the macrocomplexes **PANb** and **PSSNb** is likely to be due to the additional stability imparted to the compounds by the polymeric support through immobilization **(Table 6.1 entries 7, 8)**.

On comparing these values with those obtained from some of the previously reported monomeric hetero-ligand peroxo complexes of d^0 metal ions *viz.,* V and Mo, it emerged that peroxo groups attached to Nb show comparable degree of resistance with respect to pV compounds [28-33] whereas, these are several fold more resistant to catalase action than the molybdenum containing analogues [32].

Table 6.1 Catalase-dependent oxygen release from niobiumperoxo compounds

Entry	Compound	Concentration		Peroxide content (mM)	Loss of peroxide (μM/min)
		mg/mL	(mM)		
1.	**NaNb**	0.105	0.2	0.8	16.7
2.	**KNb**	0.068	0.2	0.8	24.5
3.	**NbAla**	0.065	0.2	0.6	9.1
4.	**NbVal**	0.070	0.2	0.6	11.6
5.	**NbA**	0.089	0.2	0.6	11.4
6.	**NbN**	0.079	0.2	0.6	8.6
7.	**PANb**	0.096	0.2	0.6	7.1
8.	**PSSNb**	0.123	0.2	0.6	7.5
9.	**DPV**[a]	0.034	0.2	0.4	12.0
10.	**DMo_1**[a*]	0.054	0.2	0.4	37.6

[a]Ref [32].

[*]Amount of catalase = 10 μg/mL.

On the basis of the peroxo groups present in these complexes, total peroxide loss was expected to be 0.6 mM for the triperoxo compounds and 0.8 mM for tetraperoxo compound from a solution of 0.2 mM compound concentration. We have previously observed that the catalase action on compounds of V, Mo and W led to nearly complete loss of their peroxide content within ca. 30 min of incubation as anticipated [28-33]. In contrast, as seen in **Fig. 6.3** in case of the pNb complexes, after the initial loss of two molecules of peroxo groups of the triperoxoNb complexes, within *ca.* 30 min of incubation, a unique feature observed for each of the pNb complexes is that, the compounds continued to retain one molecule of peroxide even beyond a period of *ca.* 1 h. The formation of a monoperoxo species of Nb and its high resistance to catalase action is thus evident from our data.

It is pertinent to mention that, we have preferred to employ phosphate buffer in the present study, as the use of near neutral phosphate buffers has proven to be most satisfactory in several previous investigations dealing with peroxovanadates [32,34-36] as well as peroxo compounds of Mo(VI) [32] and W(VI) [37], studied in our laboratory. Although vanadate has been reported to react with most of the compounds used in buffer including phosphate [35,38], pV compounds are known to be fairly inert and do not react with non-reducing buffers such as phosphate [34,35,36]. It is important to note that we have also conducted similar experiments using HEPES as buffer *in lieu* of phosphate buffer (**Fig. 6.4**). The rates of catalase induced degradation of the pNb compounds derived from these experiments showed no significant variation [17.0, 11.0, 11.9 and 9.4 μM/min for **NaNb**, **NbVal (4.2)**, **NbA (4.3)** and **NbN (4.4)**, respectively] (**Fig. 6.4**) from the results obtained using phosphate buffer (**Table 6.1**), suggesting that these buffer systems have no observable influence on the stability or the degradation profile of the compounds in solution, under the employed experimental condition.

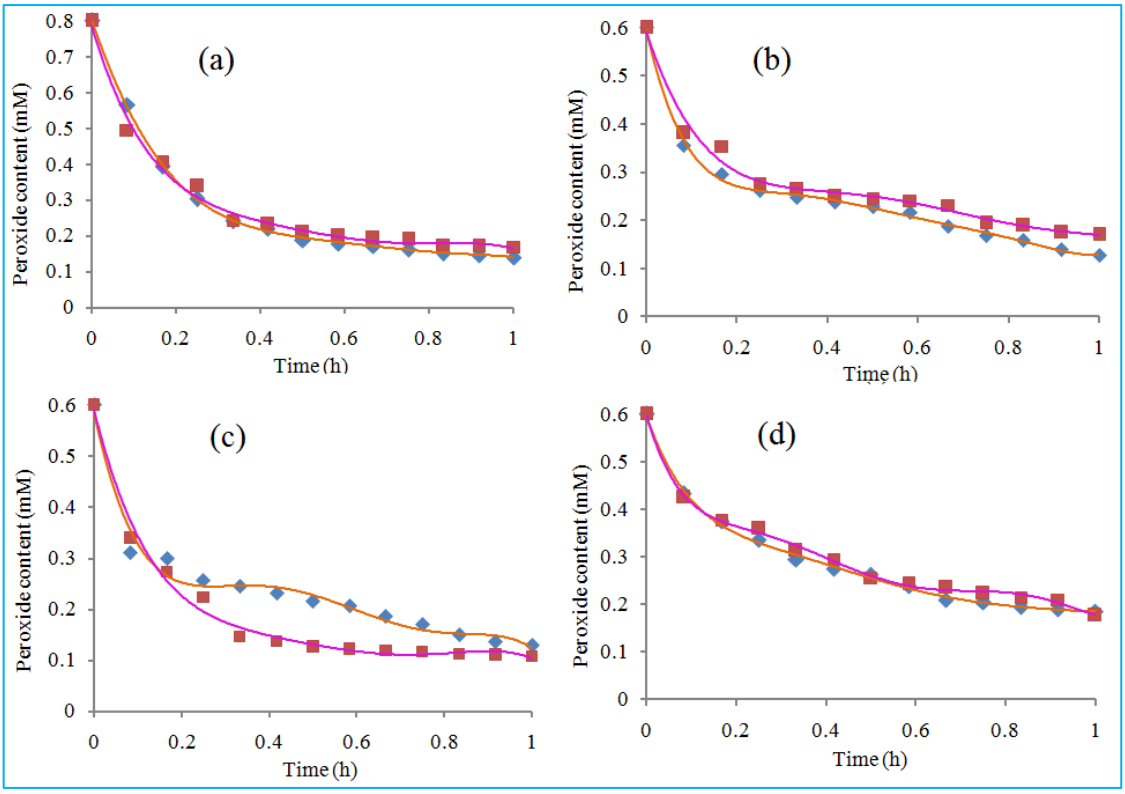


Fig. 6.4 Effect of catalase on (a) **NaNb,** (b) **NbVal (4.2)**, (c) **NbA (4.3)** and (d) **NbN (4.4)**. The test solution contained (♦) phosphate buffer or (■) HEPES buffer (50 mM, pH 7.0) and the catalase (40 μg/mL) which was incubated at 30 °C for 5 min. Compound was then added to the reaction solution, aliquots were drawn at indicated time points, and loss in peroxide content was determined.

We have further examined the nature of niobium species formed during and after the catalase action on the pNb complexes with the help of ^{93}Nb NMR spectral analysis. Presented in **Fig. 6.5** are the spectral changes taking place on incubation of **PANb** (**3.1**) with catalase. The spectrum recorded after 30 min of treatment with catalase showed the decrease in intensity of the major peak at -1530 ppm corresponding to the original pNb complex with a simultaneous increase in the intensity of anew peak at -1189 ppm which may be ascribed to the catalase induced degradation product of the pNb complex. After 2 h of the reaction, the complete degradation of peroxoniobium species in solution occurred which is evident from the disappearance of the peak at -1530 ppm and presence of a new major peak at -1127 ppm (**Fig. 6.5 c**) attributable to oxoniobate species [39].

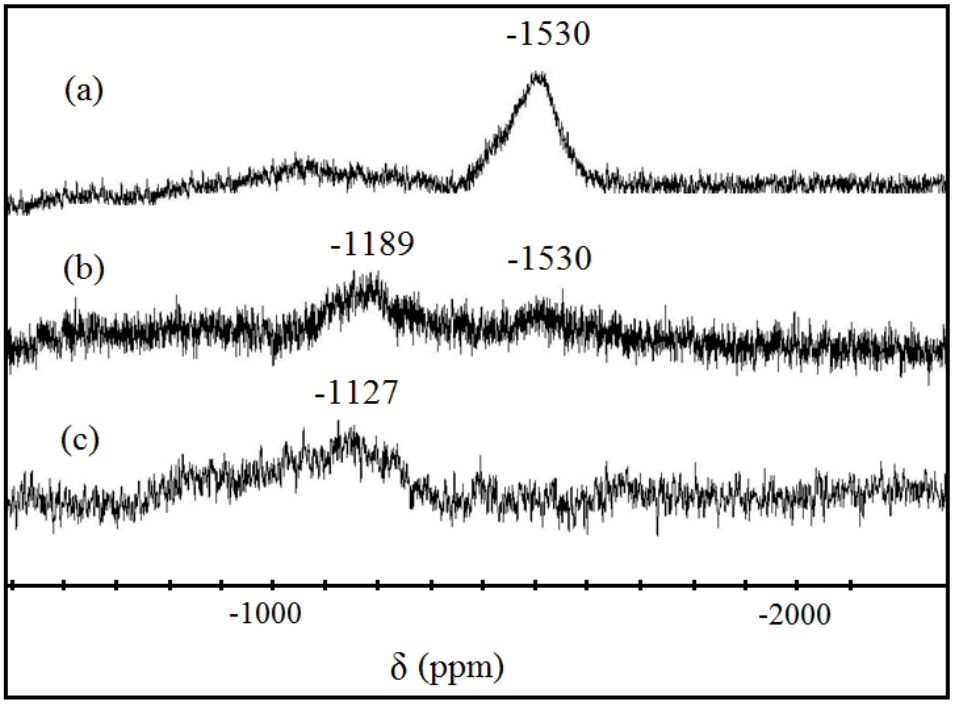


Fig. 6.5 ^{93}Nb NMR spectra of a 0.2 mM solution of **PANb**, its catalase degradation products. The spectra were recorded as follows: (a) aqueous solution of **PANb** in water immediately after preparation, (b) **PANb** (20 mM) incubated with catalase (40 mg/mL) after 30 min incubation and (c) solution of (b) 2 h later.

6.3.3 *In vitro* cell viability studies

With an aim to examine the toxicity of the pNb compounds, the viability of Raw 264.7 murine macrophage cells in the presence of each of the compounds was determined by using the MTT assay [40]. It is clear from the results presented in **Fig**. **6.6** that none of the complexes, at lower concentrations up to 20 μM, shows any significant toxic effect on the cultured macrophage cells. Among the tested complexes, only the arginine containing **NbA** appears to have a dose dependent effect on the viability of the cells. On exposure of the cells to relatively higher concentration (200 μM) of the complexes, the cell viability was found to be reduced by *ca.* 35.0% and 31.6% for **NbA** and **NbN**, respectively after 24 h, whereas the cells showed very low sensitivity to the rest of the compounds *viz.*, **NaNb**, **NbAla** and **NbVal** (**Table 6.2**).

Although the pNb compounds investigated have been observed to be minimally toxic even at high compound concentration, close analysis of the data obtained of the

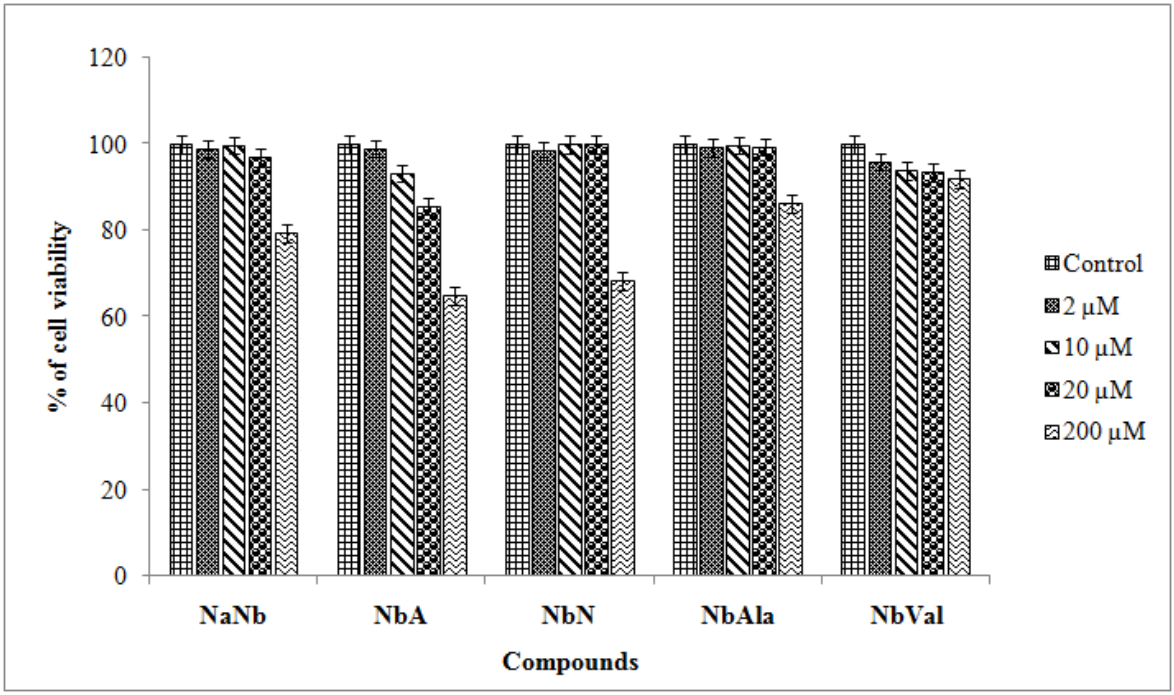


Fig. 6.6 The viability of Raw 264.7 murine macrophage cells as measured by the MTT assay. Cells were treated with the monomeric pNb compounds (**NaNb** and **4.1-4.4**) and incubated for 24 h. MTT was added to the cells and incubated for 4 h. The cell viability was assessed by measuring the absorbance at 580 nm and expressed as mean (± SE) from three separate experiments.

Table 6.2: Effect on Raw 264.7 murine macrophage cells when treated with peroxoniobium complexes at 200 μM concentration of pNb compounds

Entry	Complexes	Cells killed (%)
1	**NaNb**	20.7
2	**NbA**	35.0
3	**NbN**	31.6
4	**NbAla**	13.7
5	**NbVal**	8.1

present niobium complexes (**Table 6.2**) reveals that the coordinated ligand probably plays an important role in the observed variations in cell viability of the compounds. Further work will definitely be needed to comment on the observed trend. Nevertheless, taking into account the literature reports co-relating cytotoxicity of metal complexes with their ability to interact with DNA [41-43], it may be hypothesized that the higher cytotoxicity of **NbA** may arise from the ability of the guanidinium group of arginine, well-separated from the negatively charged coordination sphere by a long carbon chain, to interact with DNA through H-bonding leading to its stronger DNA binding affinity. The next effective peroxoniobium complex is **NbN** with nicotinate as ligand, and the heterocyclic nitrogen atom of which can engage in hydrogen bonding interaction with DNA. The complexes with alanine (**NbAla**) and valine (**NbVal**) show almost negligible cytotoxicity even at 200 μM, probably due to the absence of any DNA recognition element in the coordinated ligands.

6.4 Conclusion

In conclusion, the present experiments establish that the peroxoniobium compounds anchored to soluble polymers, as well as the free monomeric pNb complexes investigated, share the following distinctive attributes: (i) stability in solution with an ability to retain their structural integrity at acidic as well as physiological pH; (ii) resistance to undergo degradation under catalase action, being capable of partially retaining the co-ordinated peroxo groups even beyond 1 h of incubation with the enzyme; (iii) low toxicity towards murine macrophage cells. The pNb compounds thus fulfil at least a few of the most fundamental requirements for a soluble metal complex to be effective as a therapeutic or bio-relevant agent suggesting inter alia, their significance as a promising system to carry forward for *in vivo* studies.

Appendix: 6A

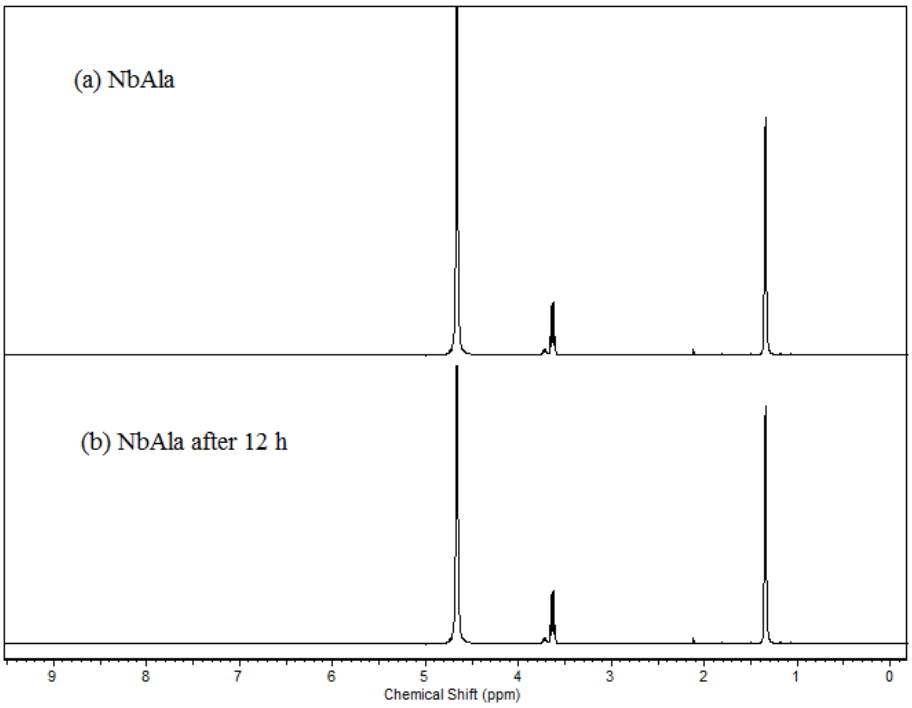


Fig. 6A.1 The ^{1}H NMR spectra of **NbAla** in D_2O. The spectra were recorded as follows: (a) **NbAla** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

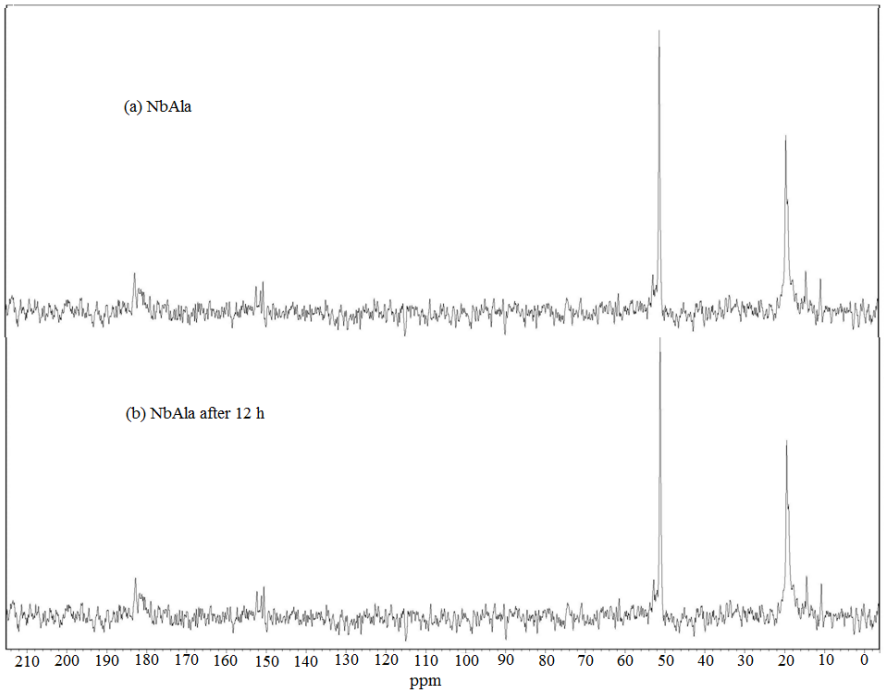


Fig. 6A.2 The ^{13}C NMR spectra of **NbAla** in D_2O. The spectra were recorded as follows: (a) **NbAla** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

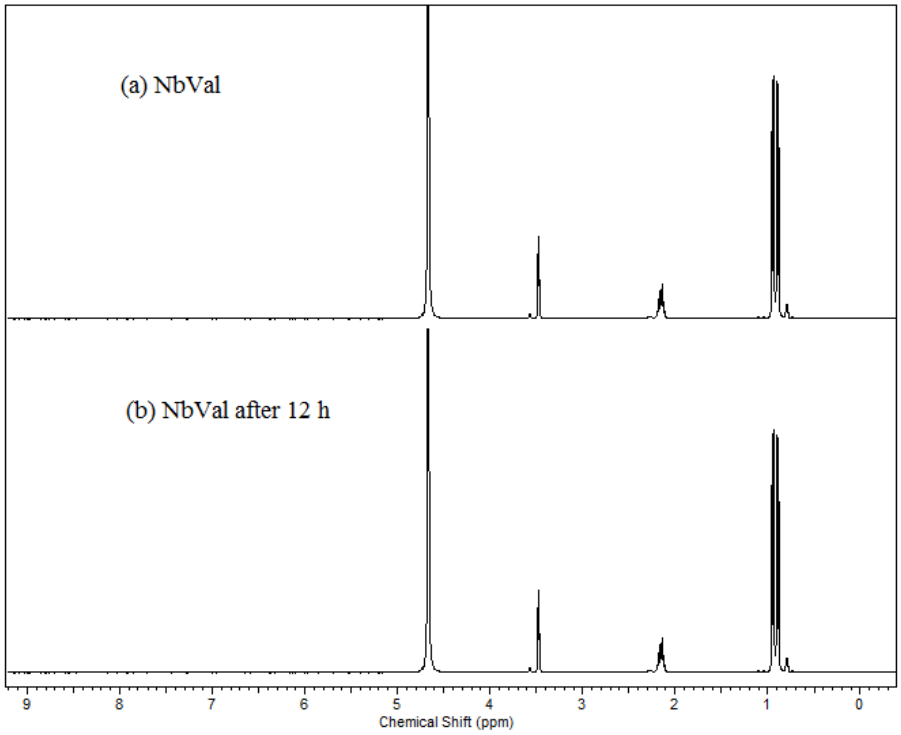


Fig. 6A.3 The ^{1}H NMR spectra of **NbVal** in D_2O. The spectra were recorded as follows: (a) **NbVal** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

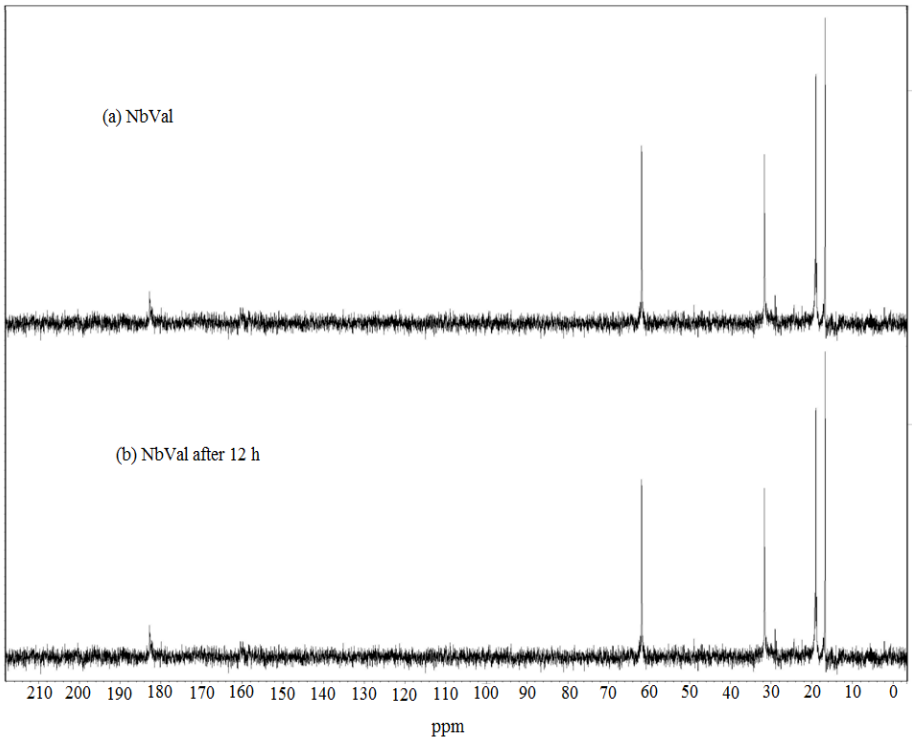


Fig. 6A.4 The ^{13}C NMR spectra of **NbVal** in D_2O. The spectra were recorded as follows:

(a) **NbVal** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

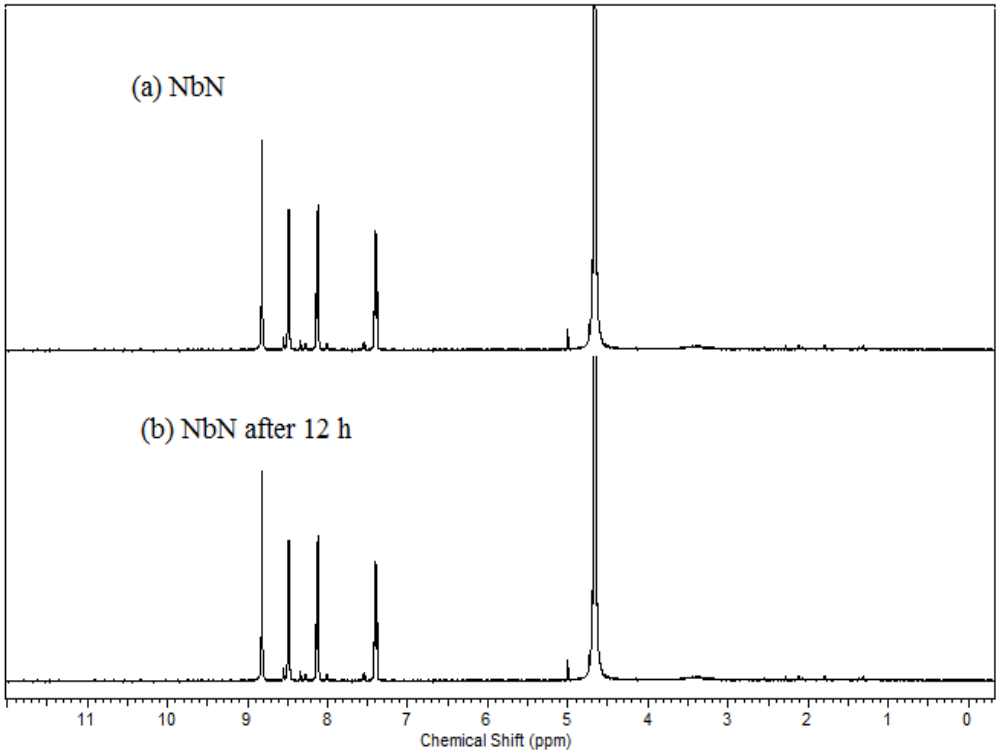


Fig. 6A.5 The ^{1}H NMR spectra of **NbN** in D_2O. The spectra were recorded as follows: (a) **NbN** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

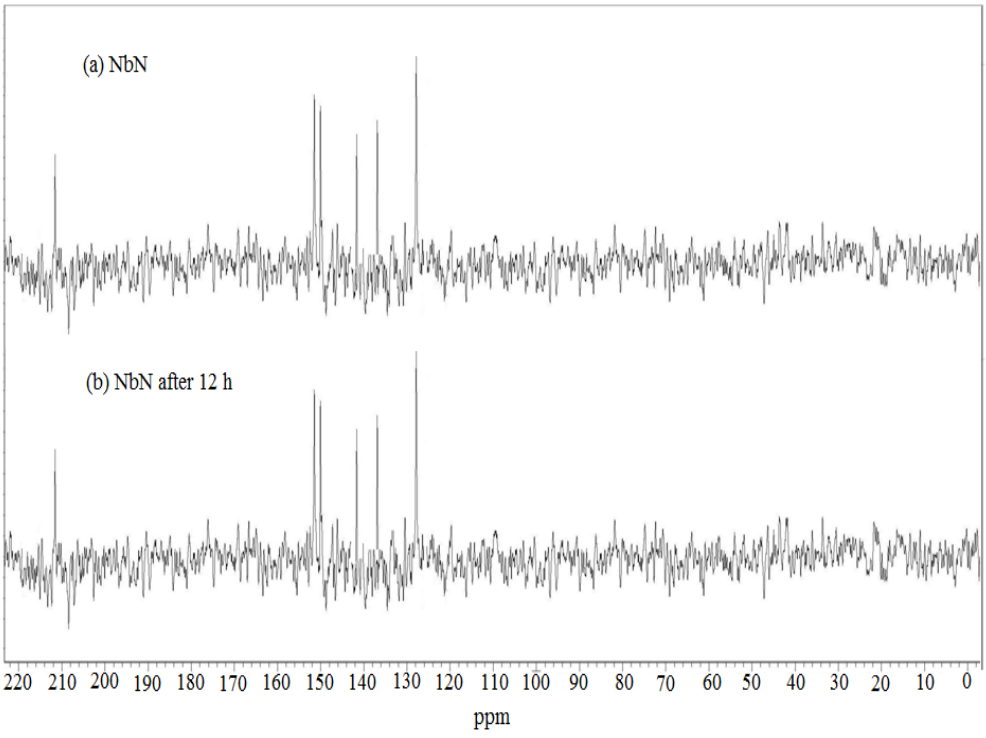


Fig. 6A.6 The ^{13}C NMR spectra of **NbN** in D_2O. The spectra were recorded as follows:

(a) **NbN** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

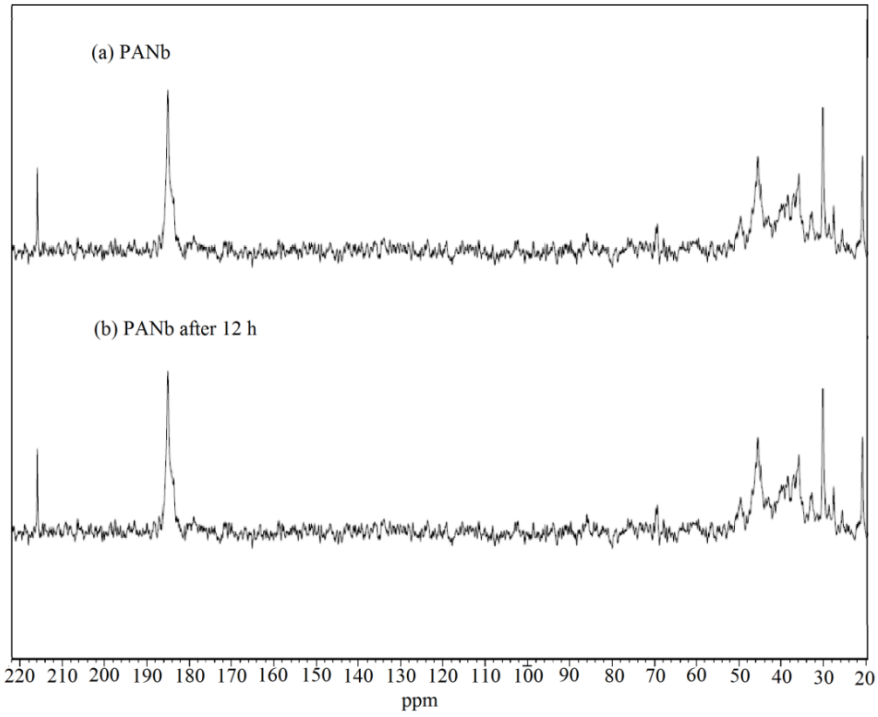


Fig. 6A.7 The ^{13}C NMR spectra of **PANb** in D_2O. The spectra were recorded as follows: (a) **PANb** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

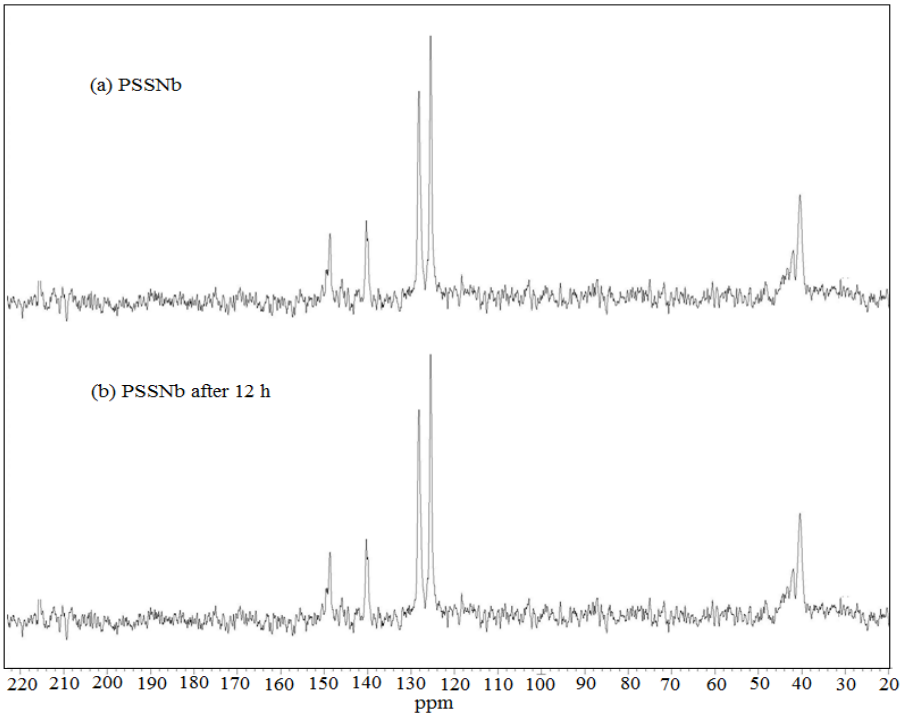


Fig. 6A.8 The ^{13}C NMR spectra of **PSSNb** in D_2O. The spectra were recorded as follows: (a) **PSSNb** in D_2O immediately after preparation, (b) solution of (a) 12 h later.

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