

Aqueous solutions of NMA, Na₂HPO₄, and NaH₂PO₄ as models for interaction studies in phosphate–protein systems

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Abstract

Phosphate buffers are essential for many areas of studies. However, their influence on buffered systems is often ignored. The phosphate salts can interact with biologically important macromolecules (e.g. proteins) and stabilize or destabilize them. With our research, we want to answer question what kind of interactions, if any, occur between phosphate ions and a protein backbone model – *N*-methylacetamide (NMA). ATR–FTIR spectroscopy in the amide I range and in the regions characteristic for P–O vibrations provides information on direct and indirect (water–mediated) interactions. The analysis is supported by chemometric, DFT, and QTAIM calculations. Our results indicate that direct NMA–phosphate ion interactions are quite rare and indirect. Water molecules seem to play an important role in such systems. The model studies indicate that no preferential interactions between NMA and phosphate ions in solutions are formed, and may imply that such interactions are also unfavorable in protein–based systems.

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1. Introduction

The constant pH of a solution is extremely important in many areas of chemistry, biology and physics. A simple way to stabilize it is to use buffers. Phosphate buffer is one of the most popular buffer in biological applications, composed of dihydrogen and hydrogen phosphate salts in various proportions. Its thermal stability and relatively wide physiological pH range ($\text{pH} = \pm\text{pK}_a$, i.e. 7.2 ± 1.0) are advantageous in many protein-involving studies.[1] Phosphates are also essential building blocks of many biologically relevant molecules: DNA, RNA, phospholipid bilayers, ATP. The stability or energetics of various phosphate-related compounds makes these ions a perfect choice from the biological point of view.[2] Ideally, buffer constituents should not be able to interact with other components of the solution. Often the Pathak [3] effect of buffers on co-solutes is considered negligible, especially if their concentration is significantly higher than the buffer concentration (20-50 mM on average). However, even if the concentration is so low, some contacts occur and affects the type of interactions in the analyzed solutions,[4, 5, 6] especially if these solutions contain proteins or other macromolecules with concentrations usually a few orders of magnitude lower.

Recently, it has been proven that despite their low concentrations the ionized molecules of buffer components may also exert the Hofmeister effects on buffered macromolecules.[7, 8, 9] Thus, the electrochemical nature of a buffer must be taken into account when solutions are being prepared, not

23 only their optimal pH range. It is proven that buffer components can in-
24 teract with polar and even non-polar fragments of a macromolecule through
25 electrostatic or dispersive forces or influence their hydration.[10, 11, 12, 13]
26 Such interactions can be either beneficial [14, 15, 16, 17, 18] or deleterious
27 [19, 20, 21] and may affect not only thermal stability but many other prop-
28 erties of macromolecules.[9, 7] According to the classical Hofmeister series,
29 HPO_4^{2-} ion can be considered as a kosmotropic agent, i.e. it orders water
30 molecules in its surrounding. However, its water-structure-making proper-
31 ties not always explains the protein salting-out effect (and also its stability
32 in solutions).[22, 23] Yet the idea of structure making or breaking can be
33 misleading because enhancement or weakening of water interactions in hy-
34 dration shells can occur simultaneously by a separation of hydration water
35 population into distinct ordered and disordered sub-populations.[24] Despite
36 the fact that the physiological role of phosphate ions is extremely important,
37 their hydration is still poorly understood.[3, 25, 26, 27, 28, 29, 30]

38 This article is focused on the influence of the *N*-methylacetamide (NMA)
39 molecule, which is often used as a minimal model compound of the pro-
40 tein backbone (a single peptide bond),[31, 32, 33, 34, 35, 36] on selected
41 phosphate salts. NMA has been widely investigated in terms of its inter-
42 actions with halide, alkali, and earth rare metals ions by means of many
43 computational and experimental methods.[37, 33, 38, 39] The possibility of
44 dimers and trimers formation of NMA molecules in solution has also been
45 confirmed.[40, 41] Various clusters of NMA with protic and aprotic solvents
46 has been described.[42, 43, 44, 45, 46] Time- or temperature-dependent
47 changes in NMA solution structures, determined mainly by means of the vi-



48 brational spectroscopy, indicate that the structure of NMA oligomers closely
49 resembles secondary structures of the protein backbone and in the pres-
50 ence of water undergoes a hydrophobic collapse similarly to more complex
51 peptides.[47, 48] From a computational point of view, NMA is also an ideal
52 minimal model for quantum mechanical calculations and molecular dynamics
53 simulations concerning protein solvation. Such an approach has been recently
54 applied to study interactions of NMA with various osmolytes and co-solutes,
55 such as DMSO, TMAO, urea, tetramethylurea, and trehalose.[49, 50, 51]
56 The choice of NMA as a model is also dictated by the greatest similarity,
57 among other peptide bond imitating amides, of its hydration to the hydra-
58 tion of protein.[52] Therefore, the use of NMA molecule in presented studies
59 appears to be justified.

60 2. Materials and Methods

61 2.1. Chemicals and Solutions

62 Dibasic sodium phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 99%, Sigma-Aldrich),
63 monohydrate monobasic sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 99%, Sigma-
64 Aldrich), NMA (99%, Aldrich), and deionized water ($<0.01 \text{ S} \cdot \text{cm}^{-1}$), were
65 used as supplied. For ATR-FTIR experimental section, ten solution series
66 of Na_2HPO_4 -NMA system were prepared. Concentration of Na_2HPO_4 was
67 kept approximately constant in each series (0.0, 0.025, 0.05, 0.075, 0.1, 0.15,
68 0.25, 0.3, 0.4, 0.5 $\text{mol} \cdot \text{dm}^{-3}$, respectively). Each of the series consisted of
69 seven different solutions in which concentration of NMA was increased (0.0,
70 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 $\text{mol} \cdot \text{dm}^{-3}$, respectively). The same method was
71 used to prepare six series of NaH_2PO_4 -NMA water solutions, where con-

72 centration of NaH_2PO_4 was approximately equal (0.0, 0.2, 0.4, 0.6, 0.8, 1.0
73 $\text{mol}\cdot\text{dm}^{-3}$, respectively), and each of this series consisted of eight solutions
74 where concentrations of NMA were equal 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0
75 $\text{mol}\cdot\text{dm}^{-3}$, respectively. Different concentration ranges were dictated by the
76 differences in phosphate salts solubility. The concentrations of all chemi-
77 cals were selected according to the demands of experimental technique. In
78 most cases, the ATR-FTIR spectroscopy requires relatively high concentra-
79 tions of solutes to obtain satisfactory spectra with low signal-to-noise ratio.
80 Additionally, water absorption bands may obscure bands of other solution
81 components, as in the case of NMA. However, as mentioned in paragraph
82 2.2.2, each concentration series was utilized to calculate derivatives or spec-
83 tra in the infinite dilution approximation. Thus, all obtained spectra may be
84 considered as corresponding to very diluted solutions.

85 All molar concentrations and molalities of NMA, water, and phosphate
86 salts for the above-mentioned systems were calculated basing on appropri-
87 ate weights of all components and densities of all prepared solutions, which
88 were measured by means of a U-shaped tube densitometer (MG-2, UniLab,
89 Poland). pH of each sample was measured with the Handylab pH-meter
90 (Schott) equipped with a pH microelectrode. The average pH of samples
91 containing Na_2HPO_4 or NaH_2PO_4 was equal to 9.21 ± 0.21 or 4.50 ± 0.35 , re-
92 spectively. Such values indicated that in those solutions only one form of
93 phosphate ion was predominant ($>97\%$). [25] Spectroscopic studies also con-
94 firmed that only one spectroscopic form of phosphate ions was present in
95 each solution.

96 The bulk water spectrum was subtracted from each measured spectrum



97 with a subtracting factor calculated on the basis of molar concentration. That
98 way, a grid of NMA–phosphate concentrations and corresponding spectra was
99 created (i.e. a so-called three-way data set with four dimensions: wavenum-
100 ber, NMA concentration, phosphate salt concentration, and absorbance).
101 Due to the inevitable instrumental and preparation error, concentrations of
102 all solution components in selected concentration dimension were not ex-
103 actly equal. A fixed concentration of a spectral species in a grid line (i.e.
104 a selected concentration dimension) is crucial for the next step of spectra
105 analysis. Thus, the set of spectra was used for spectra interpolation to the
106 grid of evenly spaced concentrations of NMA or phosphate ions using the
107 Gridfit tool for Matlab by John R. D’Errico. The interpolation scheme was
108 set to “bilinear” with a recommended smoothing factor of 1.

109 *2.2. Methods of interaction studies in protein-osmolyte-water systems*

110 *2.2.1. ATR–FTIR spectroscopy*

111 Spectra of all prepared solutions were recorded on Nicolet 8700 FTIR
112 spectrometer (Thermo Scientific, Waltham, MA) equipped with an ATR
113 accessory (6 internal reflections Ge crystal, Specac Ltd., Orpington, Great
114 Britain). Temperature was kept at 25.0 ± 0.1 °C using an electronic tem-
115 perature controller (Specac Ltd., Orpington, Great Britain). Each recorded
116 spectrum was obtained by measuring and averaging of 128 independent scans
117 with 2 cm^{-1} resolution. The spectrometer was purged with dry nitrogen to
118 minimize the influence of water vapor and carbon dioxide. All ATR–FTIR
119 spectra were analyzed using commercial software: GRAMS/32 (Galactic In-
120 dustries Corp., Salem, NH), OMNIC (Thermo Scientific, Waltham, MA),
121 and Matlab (the MathWorks, Natic MA).



122 2.2.2. *Difference spectra method*

123 The difference spectra method applied in our research is based on the
124 method proposed and developed by Stangret *et al.*[53, 54, 55, 56, 52] In
125 general, the method allows to isolate the spectrum of an individual affected
126 by some external factor (e.g. the presence of a co-solute, temperature, a
127 change in concentration). The difference between the “bulk” (unaffected)
128 spectrum and the one corresponding to the external change, i.e. a spectrum
129 carrying all information about changes in such a system, can be added to
130 the “bulk” spectrum with an appropriate coefficient. The coefficient carries
131 information on the affected number, N , i.e. the number of moles of the
132 analyzed individual perturbed by one mole (or one unit) of the affecting
133 agent. However, the affected spectrum cannot be calculated if the affected
134 number is unknown. The task of simultaneous determination of the number
135 and affected spectrum is not trivial, and some ways to achieve the goal are
136 presented in our previous papers.[57, 55]

137 Instead of a simple spectra difference calculation (where one spectrum
138 corresponds to a unaffected species and the second one to the same species
139 perturbed by an affecting agent or factor), a series of spectra with a fixed
140 concentration of spectral species of interest is measured. In each spectrum
141 of the series the molality, m_a , of an affecting agent (or factor) varies. From
142 such a series for each wavenumber a derivative of absorbance vs. m_a is then
143 calculated. The derivative at $m_a = 0$ (i.e. the extrapolation of the change
144 in absorbance to the infinite dilution of the affecting agent):

$$D_\nu = \left(\frac{\partial A_\nu}{\partial m} \right)_{m \rightarrow 0}$$

145 gives an information on changes caused by the introduction of affecting agent,



146 similarly to a simple difference spectrum. Such a derivative is calculated
147 for each wavelength, (we will denote it as D), thus a set of all calculated
148 derivative values has a spectrum-like nature. In fact, its shape contains
149 only the information on changes in the spectral shape of analyzed species of
150 interest caused by the introduction of affecting agent or factor.

151 2.2.3. DFT and QTAIM calculations

152 DFT calculations of simple NMA–H₂O–phosphate ion systems were per-
153 formed according to the scheme presented in our previous paper.[24] The
154 final structures and frequencies were calculated with the M06-2X density
155 functional method,[58] the aug-cc-pVTZ basis set,[59] and the conductor-like
156 polarizable continuum model (CPCM), with water set as the solvent.[60, 61]
157 The basis set superposition errors of energies (BSSE) were calculated for the
158 final CPCM-optimized geometries using the counterpoise method.[62] Such
159 a scheme of calculations allowed us in our previous paper to reproduce and
160 interpret changes in the frequencies of vibration of simple complexes to a
161 satisfactory degree.[24] In order to compare the energy values of hydrogen
162 bonds, the energies of simple water clusters composed of up to 4 molecules
163 of water were also calculated.

164 In our research, almost only the *trans* form of NMA was taken into consid-
165 eration. In aqueous solution, the *trans* conformation is much more probable,
166 as confirmed by experimental results and theoretical calculations.[63, 64, 65]
167 Moreover, it reflects better the conformation of the protein backbone.

168 Due to a significantly large number of atoms, the geometry optimizations
169 of hydrated complexes were performed only in the gas phase using the same
170 method, but with a smaller basis set – cc-pVTZ. Electron densities of such

171 large systems, calculated for the optimized final structures, were next ana-
172 lyzed according to the Quantum Theory of Atoms In Molecules (QTAIM).[66]
173 Energies of selected hydrogen bonds were estimated [67] on the basis of the
174 potential energy density, V_r , at the bond critical points between hydrogen
175 atom and its acceptor:

$$E_{HB} = \frac{1}{2}V_r$$

176 However, the calculated E_{HB} values were used only for comparison between
177 various systems.

178 The optimization and frequency calculation steps were performed with the
179 Gaussian 09v.D1 (Gaussian Inc., Wallingford, CT) software [68] available at
180 the Academic Computer Center in Gdansk (TASK), analyzed and visualized
181 with Avogadro software v.1.1.1.[69] Calculations within the QTAIM theory
182 were performed with the Multiwfn software v. 3.3.9.[70]

183 **3. Results and Discussion**

184 *3.1. Initial remarks*

185 In our initial remarks we need to justify measurements and analysis of
186 spectral series of NMA (or phosphate ions) affected only by its concentration
187 (i.e. in such systems only NMA, or phosphate ions, and water are present).
188 Changes in such spectral series are characteristic only for direct or indi-
189 rect interaction between the same kind of molecules. Such interactions have
190 to be taken into account in ternary systems (i.e. NMA–water–phosphate
191 ion) because the presence of an additional solution component may promote
192 concentration–dependent structural changes of the analyzed molecule. The



193 addition of the third component of the solution causes decrease in water con-
194 centration, and even if the concentration of the first main component (here
195 NMA) is constant with and without the third compound, the amount of ac-
196 cessible water molecules must be lower. However, in the case of our systems
197 such an effect turned out to be negligible and no concentration-dependent
198 changes in ternary systems were observed for any of analyzed components
199 (NMA, H_2PO_4^- , HPO_4^{2-}).

200 Because most of the changes in the analyzed systems in the following
201 paragraphs are very small and may not be easily visible even in the so-called
202 affected spectra, some of them will be indicated in the derivative D , which
203 carries all information about changes in the spectral series. The sense of the
204 derivative is discussed in paragraph 2.2.2.

205 *3.2. Changes in the shape of NMA spectra of pure NMA solutions*

206 Spectra of NMA affected by changes in its concentration were measured
207 in both analyzed systems of phosphate ions. Changes in such spectral series
208 were identical.

209 Molar ATR-FTIR spectra of NMA in aqueous solutions corresponding
210 to different NMA concentrations are shown in Figure 1a. The bulk water
211 spectrum was subtracted from each of them with an appropriate subtraction
212 factor. The maximum of the peak at ca. 1625 cm^{-1} , assigned to the stretching
213 vibration of C=O bond, significantly shifts towards higher wavenumbers,
214 along with the increase of NMA concentration. Changes in the position of the
215 band corresponding to the bending vibration of N-H bond, with maximum
216 at ca. 1580 cm^{-1} , are barely visible, because it is obscured by a stronger
217 C=O band. Any shift in its position is below the measurement resolution.

218 Thus, it should be concluded that this band does not change its position.
219 The confirmation of this two above-mentioned statements can be found in
220 the shape of the derivative (Figure 1b) for changes of NMA caused by its
221 increased concentration. The characteristic shape indicates a strong shift
222 towards higher wavenumber values of a single band in the amide I region.
223 Most importantly, there is no change of the band shape contour at the N–H
224 bond region. However, DFT calculation clearly indicate that the change in
225 C=O bond of NMA, being a result of interaction with another molecule, has
226 to have an influence on the N–H bond in peptide moiety, and *vice versa*.
227 For example, in a complex of two molecules of NMA (Figure 2e), theoretical
228 calculations predict that bands corresponding to the stretching vibration of
229 C=O bond and bending vibration of N–H bond split into two low- and high-
230 wavenumber components (Table 2).

231 The chemometric analysis of the spectra series clarified the picture. From
232 the concentration-affected NMA spectra three principal factors could be ex-
233 tracted (Figure 1a). The Malinowski's spectral isolation factor analysis al-
234 gorithm [71, 72] allowed to estimate band shapes of those three factors. Two
235 of them closely resembled the NMA bulk spectrum and revealed the missing
236 shift in the N–H band position, hidden in the series of spectra and in the
237 derivative D . In one of those factors both peaks (C=O stretching and N–
238 H bending) are relatively close to each other, while in the second one both
239 of them are clearly separated and shifted in directions predicted by DFT
240 calculations. As expected, these two factors exchange their relative concen-
241 trations in the spectral series (Figure 1c). Although both factors are abstract
242 mathematical representations of the gradual shifts in the spectra series, they



243 allow to conclude that observed changes were results of direct NMA–NMA
244 interactions in solution. The third isolated principal factor is similar to a
245 difference spectrum and can be simply a correction due to the change in the
246 C=O band intensity.

247 In conclusion, ATR–FTIR studies indicate that NMA in aqueous solutions
248 can form higher–order structures in the given concentration, relatively wide,
249 range. Theoretical calculations confirm these observations. Other reports
250 confirm such finding, although such interactions are thought to be weak.[73,
251 74]

252 *3.3. NMA in the presence of phosphate ions*

253 The shape and character of changes in the derivatives D of phosphate-
254 affected NMA in both considered systems were similar to the changes of
255 bands corresponding to bending vibrations of water molecules affected by
256 phosphate ions. This indicated that the main changes observed in these
257 derivatives were not caused by the phosphate-affected vibrational structure
258 of NMA. Most of the observed changes came from the water disordered by the
259 presence of phosphate ions. Thus, there was no need to isolate the spectra
260 of NMA affected by presence of phosphates, because with the given spectral
261 resolution and experimental error no changes in NMA structure could be
262 indicated and it could be even misleading. Summarizing, the vibrational
263 structure of NMA was generally not affected by interactions with phosphate
264 ions.



265 3.4. *Changes in the band shape of $H_2PO_4^-$ ion spectrum caused by an increase*
266 *of its concentration*

267 The ATR-FTIR molar spectra of $H_2PO_4^-$ ion in the P-O vibration range
268 affected by changes in its concentration are shown in Figure 3a. The bulk
269 water spectrum was subtracted from all the spectra with a subtraction factor
270 calculated according to the water molar concentration. The peak at ca. 1160
271 cm^{-1} , which can be attributed to the asymmetric stretching vibrations of P-
272 O bond,[75] slightly shifts to lower wavenumbers along with the increase of
273 $H_2PO_4^-$ concentration. There is no visible change in the band position of
274 symmetric stretching P-O vibration band at ca. 1075 cm^{-1} ,[75] except for
275 the change of its intensity. However, the shape of derivative in this region
276 suggests that the band increases its width and slightly shifts towards lower
277 wavenumbers. The blue shift of the peak at ca. 940 cm^{-1} , which can be
278 attributed to asymmetric stretching vibrations of P-OH bonds, is smaller
279 than the resolution of measurement, but the shape of the difference derivative
280 spectrum (Figure 3c) in the region of P-OH stretching vibrations suggests,
281 that the difference between spectra is meaningful.

282 The DFT calculations indicate that the complex of two $H_2PO_4^-$ ions, bind-
283 ing each other with three hydrogen bonds (Figure 2j), is even more favorable
284 than the complex of the same phosphate anion and water molecule (Table
285 1). The calculations predict the direction of changes in the wavenumbers of
286 all considered bonds of $H_2PO_4^-$ ion (Table 2) in such a complex. Thus, it is
287 possible that $H_2PO_4^-$ ions create dimers in aqueous solution.

288 Observed changes are getting stronger with the increase of concentration,
289 mostly because the probability to interact two ions in solution is higher. The



	E(el.+ZPC) ^a	BSSE ^b	ΔE^c	HB ^d	E_{HB}^e
	hartree	kJ·mol ⁻¹	kJ·mol ⁻¹		kJ·mol ⁻¹
Water (H ₂ O)	-76.415493	–	–	–	–
(H ₂ O) ₂	-152.834425	0.3	-8.8	1	-8.8
(H ₂ O) ₃	-229.255430	0.9	-22.6	3	-7.5
(H ₂ O) ₄	-305.680529	1.6	-47.2	4	-11.8
a) <i>cis</i> -NMA	-248.417519	–	–	–	–
b) <i>trans</i> -NMA	-248.420800	–	–	–	–
c) H ₂ PO ₄ ⁻	-643.742814	–	–	–	–
d) HPO ₄ ²⁻	-643.269087	–	–	–	–
e) NMA–NMA	-496.849311	1.2	-19.1	1	-19.1
f) NMA _(N-H) –H ₂ O	-324.840363	0.6	-10.1	1	-10.1
g) NMA _(C=O) –H ₂ O	-324.842722	0.5	-16.3	1	-16.3
h) NMA–H ₂ PO ₄ ⁻	-892.173602	1.4	-24.9	1	-24.9
i) NMA–HPO ₄ ²⁻	-891.704366	1.5	-36.5	1	-36.5
j) H ₂ PO ₄ ⁻ –H ₂ PO ₄ ⁻	-1287.517235	2.6	-80.4	3	-26.8
k) H ₂ PO ₄ ⁻ –H ₂ O	-720.167828	0.8	-24.2	2	-12.1
l) HPO ₄ ²⁻ –H ₂ O	-719.699748	0.9	-39.0	2	-19.5
m) NMA–H ₂ O–H ₂ PO ₄ ⁻	-968.598946	2.1	-50.1	3	-16.7
n) NMA ^f –HPO ₄ ²⁻ –H ₂ O	-968.127854	2.5	-65.1	2	-32.5

Table 1: Results of DFT (M06-2X/aug-cc-pVTZ, CPCM) energy calculations for various complexes of water, NMA, H₂PO₄⁻, and HPO₄²⁻. ^a Sum of electronic and zero point energies, ^b basis set superposition error, ^c energy of interaction (BSSE included), ^d number of excessive hydrogen bonds in a complex, ^e energy of interaction per one excessive hydrogen bond, ^f geometry optimization of such a complex possible only for the *cis* form of NMA; all other complexes were optimized with the *trans* form. Lower letters next to the types of complexes correspond to Figure 2.



Experimental frequencies					
	ν_{as} P–OH	ν_{s} P–O	ν_{as} P–O	$\nu_{\text{C=O}}$	$\delta\text{N–H}$
NMA	–	–	–	1621	1579
H_2PO_4^-	943	1078	1160	–	–
HPO_4^{2-}	–	990	1080	–	–
Results of frequency calculation for various complexes					
	ν_{as} P–OH	ν_{s} P–O	ν_{as} P–O	$\nu_{\text{C=O}}$	$\delta\text{N–H}$
a) <i>cis</i> –NMA	–	–	–	1703	1525
b) <i>trans</i> –NMA	–	–	–	1710	1569
c) H_2PO_4^-	837	1109	1280	–	–
d) HPO_4^{2-}	–	978	1136	–	–
e) NMA–NMA	–	–	–	1691 ^a /1706 ^b	1610 ^a /1581 ^b
f) $\text{NMA}_{(\text{N–H})}\text{–H}_2\text{O}$	–	–	–	1703	1595
g) $\text{NMA}_{(\text{C=O})}\text{–H}_2\text{O}$	–	–	–	1690	1584
h) $\text{NMA–H}_2\text{PO}_4^-$	851	1108	1270	1695	1618
i) NMA–HPO_4^{2-}	–	998	1094	1681	1641
j) $\text{H}_2\text{PO}_4^- \text{–H}_2\text{PO}_4^-$	927	1106	1230	–	–
k) $\text{H}_2\text{PO}_4^- \text{–H}_2\text{O}$	857	1107	1262	–	–
l) $\text{HPO}_4^{2-} \text{–H}_2\text{O}$	–	973	1149/1084	–	–
m) $\text{NMA–H}_2\text{O–H}_2\text{PO}_4^-$	869	1087	1214	1689	1588
n) $\text{NMA}^* \text{–HPO}_4^{2-} \text{–H}_2\text{O}$	–	975	1078/1165	1692	1559

Table 2: Results of DFT (M06-2X/aug-cc-pVTZ, CPCM) frequency calculations for various complexes of NMA, H_2PO_4^- , and HPO_4^{2-} . All complexes containing NMA were calculated with the *trans* isomer of the compound. ^a A group engaged in the HB formation; ^b free group. * Geometry optimization of such a complex possible only for *cis* form of NMA; all other complexes were optimized with the *trans* form. Lower letters next to the types of complexes correspond to Figure 2.



290 formation of H_2PO_4^- dimers in aqueous solutions has been confirmed in other
291 works.[76, 77, 78]

292 3.5. Changes in the shape of H_2PO_4^- ion spectra caused by the presence of 293 NMA

294 The changes in the peak shapes at ca. 1160 cm^{-1} and ca. 1075 cm^{-1}
295 (Figure 3b) are caused by presence of NMA. The maximum of the spectra
296 responsible for asymmetric stretching vibrations of P–OH bond and asym-
297 metric stretching vibrations of P–O bond changing towards lower wavenum-
298 ber values. The differences are much more evident than those observed in the
299 case of H_2PO_4^- spectra caused by the increase of its concentration. This sug-
300 gests that H_2PO_4^- ions are affected to some extent by the presence of NMA.
301 Differences in the shape of the derivatives for this NMA–affected series, and
302 the previous one, caused only by the change in its concentration, supports
303 the statement (Figure 3c).

304 The number of ions affected by NMA (Table 3) is very low ($N=0.01$ –
305 0.15) indicating that such interactions are no numerous, yet its higher than
306 in the case of the second phosphate ion. Additionally, the affected number is
307 roughly proportional to the increase of H_2PO_4^- ion concentration suggesting
308 that such weak interactions are not preferentially created or inhibited in a
309 solution.

310 The DFT calculations of simple complexes indicate that the structure
311 consisting of NMA molecule and H_2PO_4^- ion mediated by one water molecule
312 (Figure 2m) is favorable (Table 1). The results of calculations also predict
313 the direction of shifts in wavenumbers (Table 2) for this kind of structure.
314 Thus, it is likely that in such systems indirect interactions through water

$[\text{H}_2\text{PO}_4^-]$	N	$[\text{HPO}_4^{2-}]$	N
$\text{mol}\cdot\text{dm}^{-3}$		$\text{mol}\cdot\text{dm}^{-3}$	
0.1104	0.02	0.0477	0.01
0.2209	0.05	0.0954	0.02
0.3313	0.07	0.1431	0.02
0.4417	0.09	0.1908	0.03
0.5521	0.10	0.2385	0.03
0.6626	0.12	0.3339	0.01
0.7730	0.13	–	–
0.8834	0.15	–	–

Table 3: Affected numbers, N , denoting the number of phosphate ion moles affected by one mole of NMA, determined for systems in which phosphate ion concentration was kept constant in a series and NMA concentration varied.

315 molecules are preferred.

316 In sum, DFT calculations indicate, that it is possible for dimers of H_2PO_4^-
317 to occur in aqueous solution of NaH_2PO_4 . The interaction energy (Table
318 1) for this structure is more favorable than for complex consist of NMA-
319 $\text{H}_2\text{O}-\text{H}_2\text{PO}_4^-$. However, a structure composed of NMA, H_2O and H_2PO_4^-
320 molecules is also favorable in aqueous solution, which is confirmed by chang-
321 ing in spectra of H_2PO_4^- caused by presence of NMA. Finally, the most proper
322 assumption is that both of mentioned forms are present in aqueous solution

323 3.6. *Changes in the band shape of HPO_4^{2-} ion spectrum caused by the increase*
324 *of its concentration*

325 The changes in ATR–FTIR spectra caused by the increase of HPO_4^{2-}
326 concentration are shown in Figure 4a. Peaks at ca. 1080 cm^{-1} and at ca. 990
327 cm^{-1} can be assigned to the asymmetric stretching vibration of P–O bond and
328 the symmetric stretching vibration of P–O bond, respectively.[75] No changes
329 in the band shape for these two regions are visible. Therefore, HPO_4^{2-} ions
330 in aqueous solution do not interact with each other or do not exert any
331 significant influence on neighboring anions in the given concentration range.
332 Confirmation of these statements is given in the shape of the derivative D
333 (Figure 4c) which is very noisy and does not indicate any significant changes
334 in the considered spectral region. The simplest explanation of this behavior
335 is the high electrostatic charge of HPO_4^{2-} ions, which causes their repulsion in
336 aqueous solution. It was impossible to finish the optimization step of DFT
337 calculations scheme for the complex consisting of two HPO_4^{2-} molecules,
338 because the distance between this two ion was constantly increasing. Thus,
339 in contrast to H_2PO_4^- , HPO_4^{2-} ions do not form dimers in aqueous solution,
340 at least not in the analyzed concentration range.

341 3.7. *Changes in the shape of HPO_4^{2-} ion spectra caused by presence of NMA*

342 Spectra of HPO_4^{2-} ion affected by the presence of NMA are shown in
343 Figure 4b. The maxima of peaks in the regions responsible for asymmetric
344 stretching vibrations of P–O bond and symmetric stretching vibrations of
345 P–O bond shift towards lower wavenumbers. This suggests that HPO_4^{2-}
346 ions are affected by the presence of NMA in solution. However, the shape
347 of the derivative D in NMA–affected spectra series of HPO_4^{2-} ion (Figure

348 4c) is similar to spectra of HPO_4^{2-} in this series. It means, that changes in
349 the vibrational structure of HPO_4^{2-} ion being a result of interaction between
350 HPO_4^{2-} and NMA molecules are very weak and non specific.[79]

351 DFT calculations suggest that a simple structure consisting of NMA and
352 HPO_4^{2-} ion (Figure 2i) is favorable in aqueous solution (Table 1), However,
353 such a structure does not take into account the presence of water molecules.
354 It was impossible to optimize geometry of a simple complex of NMA, water
355 molecule, and the phosphate ion in the same manner as in the case of NMA–
356 H_2O – H_2PO_4^- . A strong interaction (Table 1) between NMA and HPO_4^{2-}
357 was formed with the water molecule attached on the other side of the ion
358 (Figure 2n). In a real solution, NMA in such a complex should exhibit a
359 visible shifts in its spectra (Table 2), however, in reality NMA molecules are
360 not affected significantly in the solution of HPO_4^{2-} . Thus, we can conclude
361 that such a complex is not likely to be present in solutions. Moreover, the
362 value of affected number (Table 3), which denotes the number of moles of the
363 HPO_4^{2-} affected by 1 mole of NMA molecules, is extremely low ($N=0.01$ –
364 0.03). Moreover, there is no visible correlation between its value and HPO_4^{2-}
365 concentration. This all may suggest that such interactions are inhibited
366 even at higher concentrations. Again, it indicates that interactions between
367 HPO_4^{2-} ion and NMA molecule are strictly limited and are of indirect nature.

368 3.8. DFT calculations of hydrated phosphate-NMA complexes

369 Because ATR–FTIR results clearly indicated that NMA–phosphate ions
370 interactions are limited in a solution and that direct interactions between
371 them are not likely to be possible, we focused on the hydration shell of
372 phosphate ions as a possible source of observed changes in phosphate ions



373 spectra.

374 A successful attempt to simulate the influence of NMA on the minimal
375 hydration shell of the selected phosphate ions was made. The shell in both
376 cases consisted of 13 water molecules to satisfy all hydrogen bond donor and
377 acceptor sites of phosphate ions. NMA molecule, with its minimal number
378 of affected water molecules $N=3$, [52] was placed in the surrounding of the
379 phosphate hydration shell in a tangent plane facing phosphate oxygen atom
380 with the tangent point placed approximately between nitrogen and carbon
381 of carbonyl group of NMA. That way, four complexes, corresponding to four
382 oxygen atoms of phosphate ions, were created for each phosphate-NMA pair
383 and their structures were optimized.

384 In none of these complexes direct NMA–phosphate ion interactions were
385 formed. In all cases, NMA kept its water molecules and in almost every case
386 the NMA–3H₂O complex oriented itself perpendicularly to its initial orientation.
387 In Figure 5 only two selected structures are presented. Due to the
388 size of figures and the same most important features, all other structures
389 are presented in Supplementary Materials. In the H₂PO₄[−]–based systems, a
390 water molecule bridging the N–H bond and the phosphate hydration shell
391 was not incorporated into the hydration layer of H₂PO₄[−] in none of the optimized
392 complexes (Figure 5a), while in the case of HPO₄^{2−} the molecule was
393 forced in two cases to interact directly with the phosphate ion, reshaping
394 its hydration shell (Figure 5b). We calculated oxygen-oxygen distances of
395 water molecules in hydration shells, R_{OO} , of both ions for all those systems
396 (Figure 6). Results indicate that although water molecules around H₂PO₄[−]
397 ion are on average marginally affected by the presence of NMA yet some

398 of R_{OO} distances are shortened, which can be a sign of an enhancement of
399 water structure in such a binary system. In the case of HPO_4^{2-} the R_{OO}
400 distances were significantly larger when NMA was introduced to the system.
401 Such a result could suggest that the hydration shell had swelled and possibly
402 phosphate–water interactions had weakened. Instead, spectroscopic results
403 suggested that interactions between oxygen atoms of HPO_4^{2-} ion and wa-
404 ter molecules had strengthened (the PO stretching vibration bands shifted
405 towards lower wavenumbers). While results for H_2PO_4^- –water–NMA sys-
406 tem stay in a good agreement with experimental ones, DFT calculations of
407 HPO_4^{2-} –water–NMA system do not validate clearly experimental observa-
408 tions. A more reliable picture of interaction emerges from the results of the
409 QTAIM calculations and the analysis of energies at specific points in space
410 between atoms, called critical points.

411 3.9. QTAIM calculations

412 Electron densities of aqueous complexes, a “by-product” of DFT cal-
413 culations, were subjected to the analysis according to the QTAIM theory.
414 The hydrogen bond energy between donor and acceptor is proportional to the
415 potential electron density, V_r , at the critical point between proton and its
416 acceptor [67] and can be used to estimate the influence of an external fac-
417 tor on its changes. Results of the calculations of both types of complexes
418 (HPO_4^{2-} – and H_2PO_4^- –based) indicate that the presence of NMA with its
419 three water molecules influences differently the mean hydrogen bond energy
420 between oxygen atoms of phosphate ion and surrounding molecules (Table
421 4). The mean HB energy gain is larger in the case of HPO_4^{2-} (-67.3 kJ), how-
422 ever, the energy is dispersed over a larger number of such interaction, as one

	$\text{H}_2\text{PO}_4^- \cdot 13\text{H}_2\text{O}$	$\text{H}_2\text{PO}_4^- \cdot 13\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O}$	$\text{H}_2\text{PO}_4^- \cdot 13\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O}$	$\text{H}_2\text{PO}_4^- \cdot 13\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O}$	Mean
	O1 ^b	O2 ^b	O3 ^b	O4 ^b	(O1–O4) ^c
ΣE_{HB}^d	-446.1	-487.1	-485.3	-494.1	-513.4
N_{CP}^e	13	12	14	13	13.5
\bar{E}_{HB}^f	-34.3	-48.9	-34.7	-38	-38.5
$\bar{E}_{\text{HB}}(\text{O1})^g$	-20.5	-22.4	-16.8	-21.5	-21
$\bar{E}_{\text{HB}}(\text{O1H})^h$	-31.7	-69.7	-22.3	-41.4	-45.5
$\bar{E}_{\text{HB}}(\text{O2})^g$	-37.6	-51.3	-35.7	-36.8	-38.8
$\bar{E}_{\text{HB}}(\text{O3})^g$	-43	-45.7	-56.9	-43.3	-45
$\bar{E}_{\text{HB}}(\text{O4})^g$	-32.9	-60.7	-33.7	-42.3	-42.3
	$\text{H}_2\text{PO}_4^- \cdot 13\text{H}_2\text{O}$	$\text{H}_2\text{PO}_4^- \cdot 13\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O}$	$\text{H}_2\text{PO}_4^- \cdot 13\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O}$	$\text{H}_2\text{PO}_4^- \cdot 13\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O} + \text{NMA} \cdot 3\text{H}_2\text{O}$	Mean
	O1 ^b	O2 ^b	O3 ^b	O4 ^b	(O1–O4) ^c
ΣE_{HB}^d	-486.6	-534.9	-548.7	-489	-517.5
N_{CP}^e	13	12	12	12	12
\bar{E}_{HB}^f	-37.4	-44.6	-45.7	-40.8	-43.1
$\bar{E}_{\text{HB}}(\text{O1})^g$	-16.8	-17	-18.3	-16.3	-18
$\bar{E}_{\text{HB}}(\text{O1H})^h$	-75	-91.9	-99.4	-80.4	-89.2
$\bar{E}_{\text{HB}}(\text{O2})^g$	-20.4	-22.6	-18.3	-16	-19
$\bar{E}_{\text{HB}}(\text{O2H})^h$	-104	-106.7	-106.2	-104.7	-106.3
$\bar{E}_{\text{HB}}(\text{O3})^g$	-27.6	-38.4	-41.1	-30.2	-34.5
$\bar{E}_{\text{HB}}(\text{O4})^g$	-37.6	-47.3	-49	-49.6	-48.1

Table 4: Energies of $\text{PO} \cdots \text{HOH}$ or $\text{POH} \cdots \text{OH}_2$ hydrogen bonds in various phosphate–NMA complexes, calculated within the QTAIM theory. All energies are given in $\text{kJ} \cdot \text{mol}^{-1}$. Labels of oxygen atoms are as in Figure 2. ^a A system composed of phosphate ion and 13 water molecules. ^b A system composed of hydrated phosphate ion (13 water molecules) and $\text{NMA} \cdot 3\text{H}_2\text{O}$; the center of NMA molecule was oriented near the O1, O2, O3 or O4 phosphate oxygen in the initial structures, respectively; the second column gives the differences between the given energies and corresponding energies in the $\text{ion} \cdot 13\text{H}_2\text{O}$ complex. ^c Mean values of energies of O1 – O4 systems. ^d The sum of all energies of hydrogen bonds between water molecules and phosphate oxygen atoms or OH groups at bonding critical points. ^e The number of bonding critical points of such hydrogen bonds found in complex structures. ^f The mean hydrogen bond energy. ^g The mean energy of hydrogen bond involving a given phosphate oxygen atom of phosphate ion. ^h The mean energy of hydrogen bond involving a given OH group of phosphate ion.

423 additional water molecule of NMA is incorporated into the hydration shell of
424 the ion. Thus, the mean energy gain for a single hydrogen bond is larger for
425 the H_2PO_4^- -based system (-5.7 kJ) in comparison to the HPO_4^{2-} -based (-4.2
426 kJ), for which the number of HB interaction is even lower when the NMA
427 molecules is approached, according to the number of critical points found.
428 Therefore, the interactions between phosphate ions and water molecules in
429 the presence of NMA is enhanced in both cases, though the need for reshaping
430 of the hydration layer of the HPO_4^{2-} ion diminishes the energetic gain.
431 Additionally, if $\text{HPO}_4^{2-} \cdots \text{H}_2\text{O}$ hydrogen bond energies are higher, the P–O
432 stretching vibrations should lower their frequencies (as seen in experimental
433 spectra), even though the DFT-based geometric parameters indicate larger
434 R_{OO} distances between hydration water molecules.

435 The QTAIM analysis clearly indicates that O–H bond of phosphate ions
436 is the most sensitive bond for any changes in the hydration layer. The
437 $\text{POH} \cdots \text{OH}_2$ hydrogen bonds experiences much larger change in such com-
438 plexes than the $\text{PO} \cdots \text{HOH}$ ones, and its share in the overall energetic gain is
439 the largest. Such a bond is obviously more numerous in the case of H_2PO_4^-
440 ion, hence the lower mean energies of hydrogen bonds in such a case.

441 We can conclude that in our case the QTAIM-supported analysis of DFT
442 calculations gives a better and more reliable picture of interactions in solu-
443 tions.

444 4. Conclusions

445 The results of FTIR studies, supported by DFT calculations of simple
446 complexes, indicates that in pure solutions of HPO_4^{2-} no dimers or higher



447 order aggregates are formed up to the concentrations selected for this work.
448 In contrast, H_2PO_4^- ions may interact with each other in aqueous solutions,
449 although such interactions are quite weak and not numerous at selected con-
450 centration range, as indicated by low affected number values, N . Similarly,
451 oligomers of NMA can be formed in its aqueous solutions.

452 In ternary NMA–water–phosphate ion solutions new types of interactions
453 are present. Both H_2PO_4^- and HPO_4^{2-} ions react to the presence of NMA
454 molecule with changes in their spectral band shapes of the PO vibration re-
455 gion. However, both experimental results and theoretical calculations suggest
456 that such interactions are of indirect manner. This conclusion is supported
457 by the lack of any significant changes in the band shape of NMA in phos-
458 phate solutions. All changes in the amide I region of this molecules can be
459 ascribed to the changes in the shape of the O–H water bending vibration
460 band. The simplest explanation is that both phosphate and NMA molecules
461 interact through their hydration shells and such a type of interaction affects
462 mostly phosphate ions, not NMA. The fact that these compounds react dif-
463 ferently may be hidden in the differences in their hydration. Water molecules
464 in hydration shells of both phosphate ions is highly organized in comparison
465 to the bulk water,[25] and to the water affected by NMA.[52]

466 In our discussion we omit the influence of Na^+ . However, our results
467 indicate that in such systems only the phosphate part is significantly affected,
468 not NMA. Thus, any interaction of the latter with sodium ion are not visible
469 with selected experimental method and is such a case it can be regarded as
470 negligible.

471 In the context of phosphate-protein backbone interactions, our results



472 may indicate that phosphate ions are excluded from the protein backbone
473 surrounding (indirect character of interactions, low N numbers). In simple
474 systems, such an approaching of the phosphate ions to the protein's hydration
475 shell would be unfavorable and promote backbone exclusion from the phos-
476 phate surrounding (and vice versa). Without other factors such interactions
477 would be beneficial for protein stability. However, the disruption of highly
478 organized hydration shell of phosphate ions could be paid for with other types
479 of interaction, e.g. electrostatic interactions with positively charge residues
480 at the protein surface. In this context, the choice of NMA as the model of
481 protein backbone may be too simplistic and insufficient. Further studies are
482 needed involving other more sophisticated models of polypeptide backbone,
483 possibly with charged groups introducing the possibility of electrostatic in-
484 teractions. Future works may also include other buffers with pH close to 7.0
485 or phosphate salts with different counterions to help understand the role of
486 buffers and their composition on ion specific phenomena involving protein
487 stability in solutions.

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491 **6. Declaration of interest**

492 The authors declare that they have no conflict of interest.

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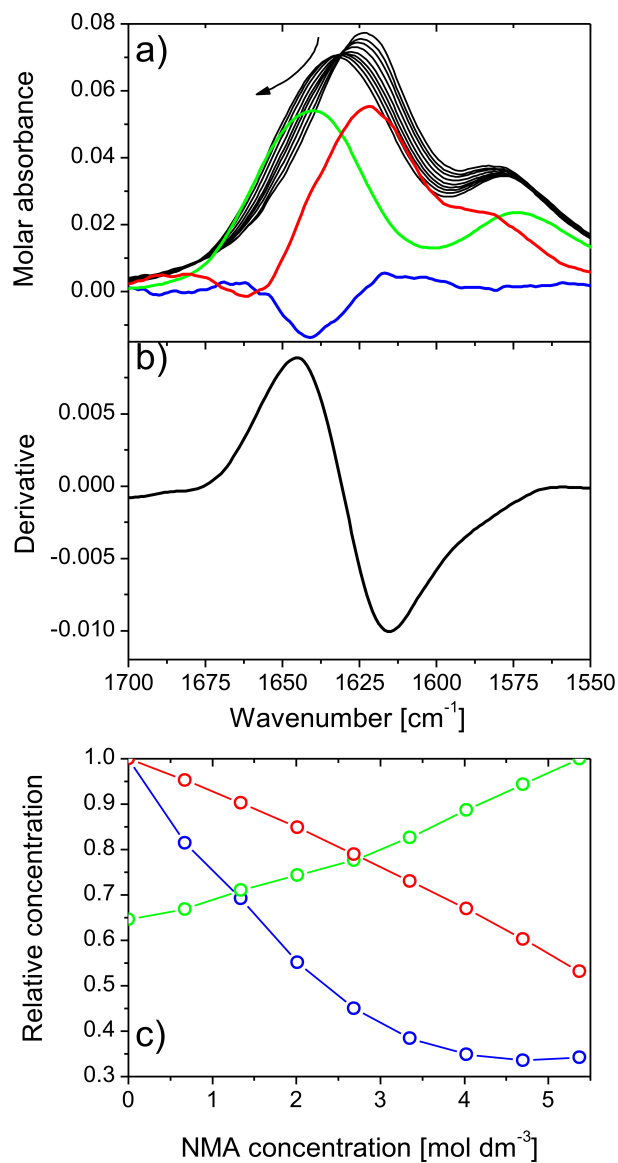


Figure 1: a) Changes in the band shape of C=O stretching and N-H bending vibrations of NMA molecule caused by its concentration increase; colors indicate first three principal factors. b) The difference derivative spectrum isolated from the series, with the band shape characteristic for a shift of a band towards higher wavenumbers. c) Relative concentrations of principal factors (colors correspond to those in Figure a).

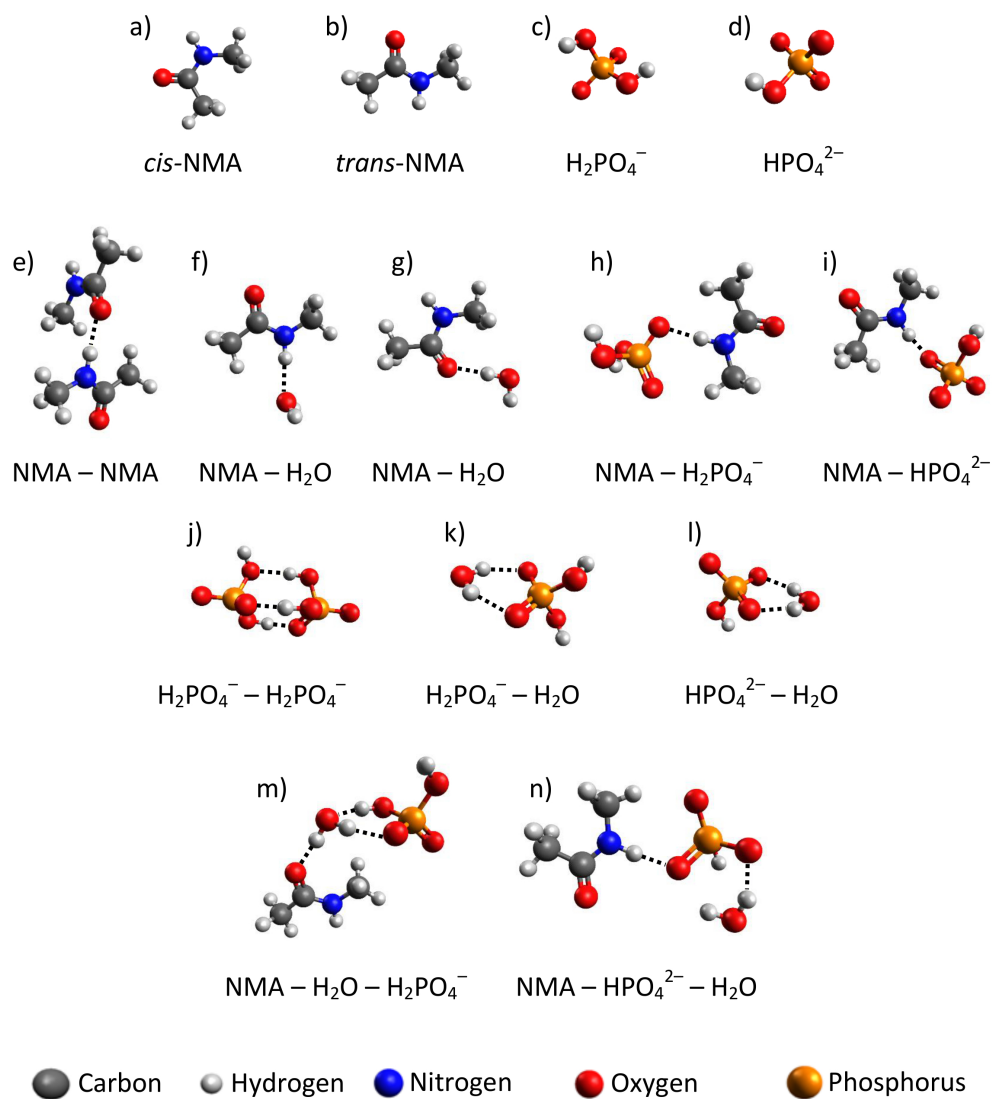


Figure 2: Optimized structures of NMA, H_2PO_4^- , HPO_4^{2-} , and their complexes. Names of these structures are the same as in Tables 1 and 2.

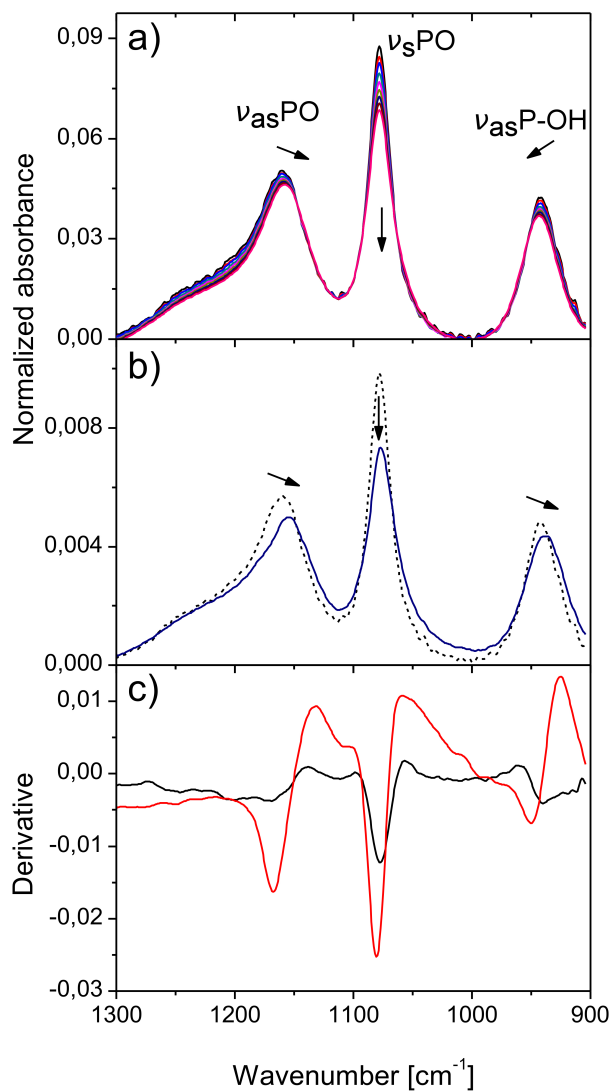


Figure 3: a) Changes in the band shape of PO vibration region caused only by the change in H_2PO_4^- ion concentration. b) the bulk (dashed) spectrum of phosphate ion and its mean spectrum affected by the presence of NMA. Arrows indicate the direction of main differences. c) The derivative D isolated from the series in figure a) (black), and the mean difference affected spectrum isolated from series of NMA-affected spectra of H_2PO_4^- (red). The meaning of the derivative is explained in paragraph 2.2.2.

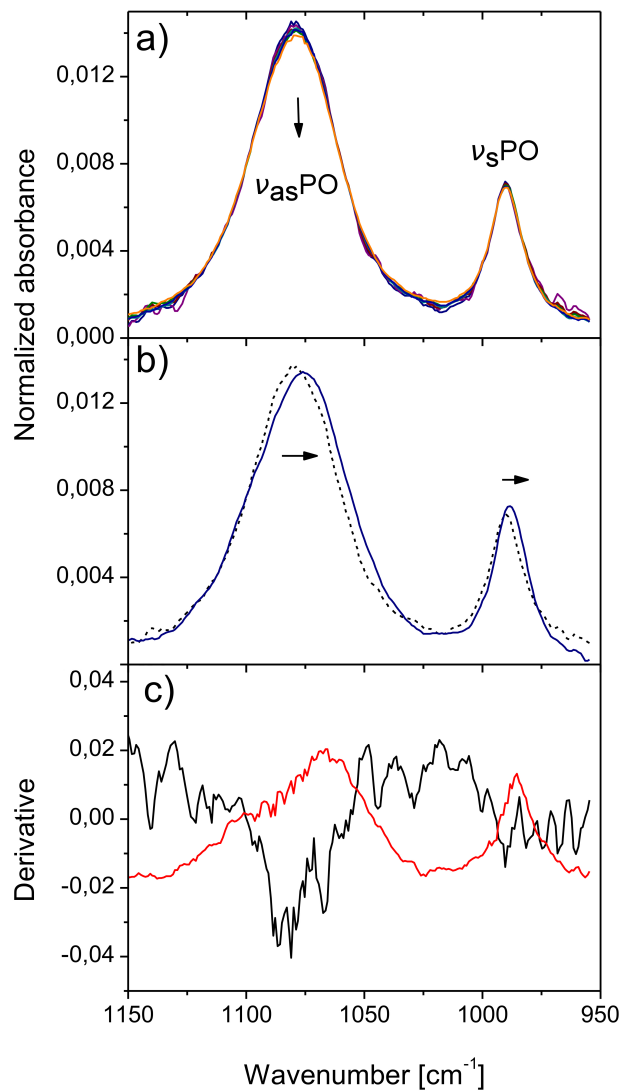


Figure 4: a) Changes in the band shape of PO vibration region caused only by the change in HPO₄²⁻ ion concentration. b) the bulk (dashed) spectrum of phosphate ion and its mean spectrum affected by the presence of NMA. Arrows indicate the direction of main differences. c) The derivative D isolated from the series in figure a) (black), and the mean difference affected spectrum isolated from series of NMA-affected spectra of HPO₄²⁻ (red). The meaning of the derivative is explained in paragraph 2.2.2.



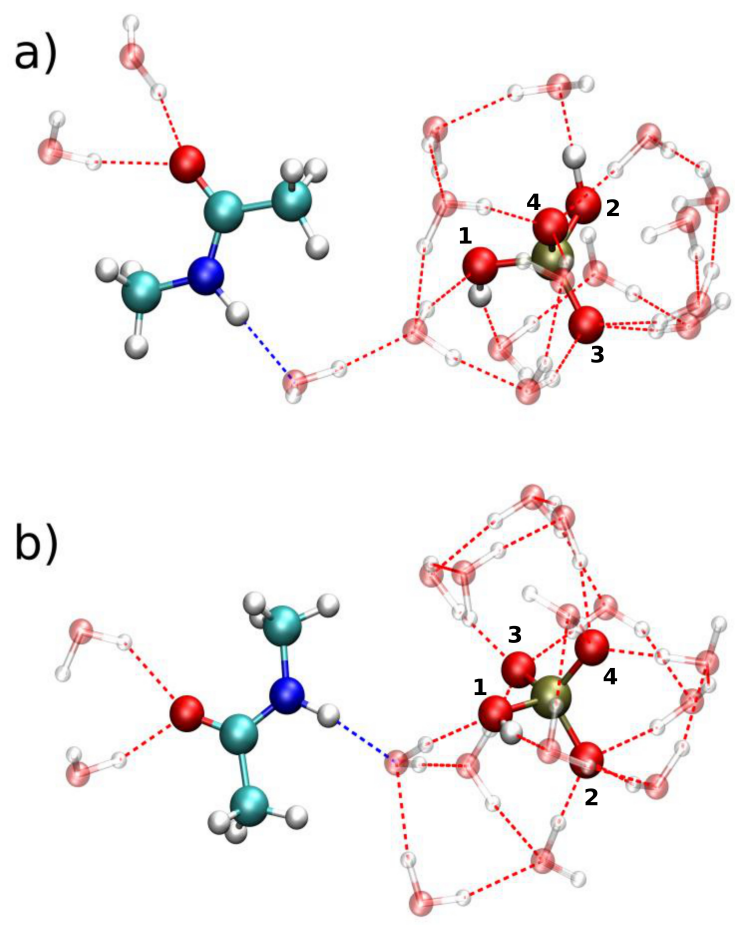


Figure 5: Selected optimized geometries of NMA-phosphate ion hydrated complexes. a) H_2PO_4^- -based system. b) HPO_4^{2-} -based system.

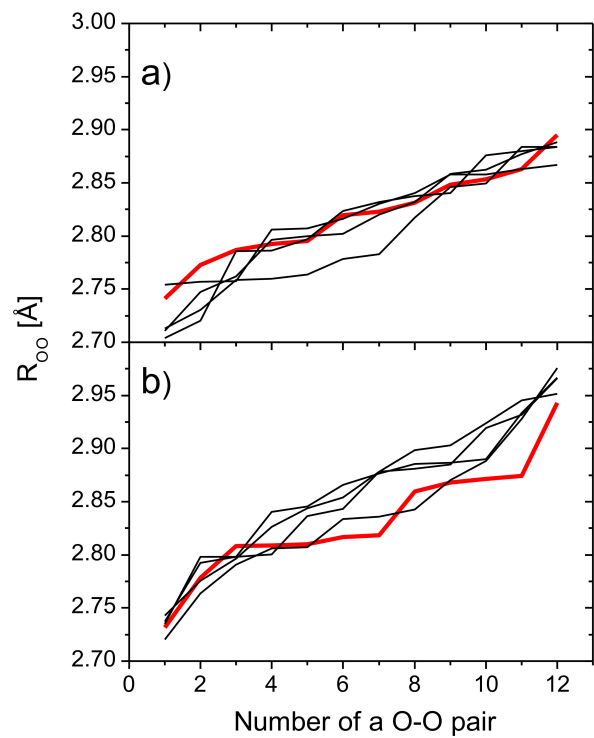


Figure 6: The distribution of oxygen-oxygen distances in the hydration layer of a) $H_2PO_4^-$ and b) HPO_4^{2-} systems. Only twelve shortest pair distances are presented. Red lines indicate systems consisting only of phosphate ion and hydration water. Black lines represents the distribution for systems including NMA molecule with its three water molecules, corresponding to four different starting NMA-ion orientations.