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Peptide dissociation in solution or bound to a polymer: comparative solvent effect

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Abstract—Dissociation of peptide when in solution or attached to a polymer was investigated. Magnified solvation of peptide-resins occurred in solvent with similar polarity. Conversely the solubilization of peptides was not usually directly related to the medium polarity. The greater the difference between acidity and basicity of solvent and its potential to form van der Waals interaction, the stronger its solubilization strength. Solvents with similar electrophilicity and nucleophilicity usually did not solvate aggregated peptide-resins nor dissolve peptides. The peptide solubilization in water-containing mixed solvents depended on combination of acidity/basicity of both components. Some criteria for choosing suitable solvents for peptide-resin solvation or peptide solubilization could be advanced.

1. Introduction

Complete understanding of the phenomenon of solute–solvent interaction has been eluding researchers for almost two centuries. Despite the exceptional relevance of this theme for all fields of science, this mystery has yet to be unravelled. The findings obtained to date only reinforce the difficulties in attaining a consensus about the rules that might govern this interaction. Amongst the innumerable factors that have been proposed over the years as controlling the solvent effect upon solute molecules, the relationship between the polarities of both components seems to be of utmost relevancy.[1] However, the so-called polarity parameter is not also easy to define or quantify and has been simply referred to as the overall solvation power of a solvent.[1,2]

In a conceptual departure from the great majority of approaches that have been applied to investigate this physico-chemical parameter, we have focused on interpreting the solute–solvent effect, deliberately using complex polymeric materials as examples of the solute component. Emphasis has been given to peptide resins and their interaction with a great number of solvents of varying polarities. This relationship has been assessed by measurement of peptide-resin solvation[3,4] or by determination of the dynamics of the interior of the solvated peptide–polymer network[5] using amino-acid type spin probes.[6] Starting from the knowledge that the presence of electrophilic and nucleophilic groups in a peptide bond (N–H and C=O moieties, respectively) might strongly affect the interaction of the solute with the solvent system, we have recently proposed the 1:1 sum of Gutman's[7] solvent electron acceptor number (AN) and solvent electron donor number (DN) as a novel, dimensionless and more accurate polarity scale.[3,4] Due to the presence of opposite concepts within the same parameter, the combined polarity term (AN+DN) was recently denoted amphoteric constant or scale.[8]

Hence, the present study aimed to pursue this approach of evaluating solvent effect upon peptide chains attached to a polymeric matrix. However, the solvent effect upon peptide chains that are free in homogeneous solution was also investigated. Needless to say, both approaches have enormous relevance. Improving the many solid-phase support processes has been crucial not only in the synthesis

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Abbreviations: Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of IUPAC-IUB (Commission on Biochemical Nomenclature (*J. Biol. Chem.* **1971**, *247* 997). Other abbreviations are as follows: AN, electron acceptor number; BHAR, benzhydrylamine resin; Boc, *tert*-butyloxycarbonyl; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DN, electron donor number; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethylsulfoxide; HF, hydrogen fluoride; HOBt, 1-hydroxybenzotriazole; HFIP, hexafluoroisopropanol; HPLC, high-performance liquid chromatography; *i*PrOH, isopropanol; MBHAR, methylbenzhydrylamine resin; MeCN, acetonitrile; MeOH, methanol; NMP, *N*-methylpiperidine; PAM, 4-(oxymethyl)-phenylacetamidomethyl-resin; PEG, poly(ethylene glycol); PIP, piperidine; TBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TEA, triethylamine; TFA, trifluoroacetic acid; TFE, trifluoroethanol; THF, tetrahydrofuran.

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of peptides[9] and other macromolecules[10–11] but also for the unique solid-phase based combinatorial strategy that allowed the generation of peptide libraries[12] and development of new therapeutic drugs through solid-phase organic synthesis.[13] In respect to the attempt at investigating solubilization and aggregation phenomenon of peptide segments in solution, the physico-chemical findings could be further extended to any other homogeneous or heterogeneous types of macromolecule interactions. Special attention might be also given to well known degenerative disorders induced by pronounced peptide aggregation in physiological conditions such as those seen in Alzheimer's,[14–16] prion-related diseases[17] and type 2 diabetes mellitus.[18]

The main assumption addressed in this work is related to the application of electron acceptor and electron donor properties, either of the solvent or of the solute components. The strategy has its origins in the findings of previous studies,[3,4] in which mixed solvents composed of strong electrophilic solvents, such as trifluoroethanol (TFE), or strong nucleophilic solvents, such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF), were unable to dissociate aggregating peptide sequence attached to a polymeric backbone. As a consequence, swelling of beads was less than that predicted by its polarity value. In this sense, we rationalize that even single solvents, when characterized by rather small differences between AN and DN values, might also be poor solvating agents, not only in peptide-resin solvation but also in dissociation of peptide chains in solution. Typically, acetonitrile (MeCN), acetone and isopropanol (*i*PrOH) would be representative of this class of solvents, as their AN/DN values are 18.9/14.1; 12.5/17.0 and 33.5/36.0, respectively.[7] The maximum difference between both properties for the three solvents is therefore not higher than 4.8 (MeCN). In complement, these two distinct peptide chain dissociation processes (peptide-resin solvation and peptide solubilization in solution) are herein evaluated in solvent systems having as great a difference as possible between acidity and basicity. In addition, other factors such as the polarity of the media, potential of the solvent to induce van der Waals interactions, effect of water and urea in the medium, pH and the strength of peptide-chain aggregation are also considered.

2. Results and discussion

2.1. Peptide chain dissociation bound to a polymeric matrix

Table 1 depicts the AN, DN and (AN+DN) values for 31 single and mixed solvents, together with the swelling degrees of the following resins: (**1**) benzhydrylamine resin (BHAR), a copolymer of styrene and 1% divinylbenzene, containing a low 0.30 mmol/g of phenylmethylamine groups;[19] (**2**) PAC-PEG-PS, a 0.18 mmol/g substituted polyethylene glycol (PEG) grafted polystyrene-1% divinylbenzene copolymer, containing the proanthocyanidin (PAC) spacer[20] (Millipore, Bedford, CA, USA); (**3**) the peptide resin $(NANP)_3$-Nle, corresponding to the immunodominant epitope of the sporozoite of *Plasmodium falciparum* malaria parasite;[21] (**4**) the peptide resin [VHHQKLVFFAEDV-amide], the 12–24 fragment[22] of the Aβ amyloid peptide responsible for formation of amyloid fibril plaques in the nervous system, inducing the appearance of Alzheimer's disease.[23] Both peptide sequences were deliberately assembled in a very highly substituted (2.6 mmol/g) methylbenzhydrylamine resin (MBHAR).[24] These last two resins have peptide contents of 82 and 72%, respectively, which will magnify the effect of peptide chains on the overall resin solvation behavior. The solvation properties of polymers were estimated by measuring, under microscopy, the swelling of dry and swollen beads[25,26] in 31 single or mixed solvents that encompass almost the entirety of the polarity scale.

Following previously established rules for polymer solvation as a function of the solvent polarity,[3,4,8] the areas of maximum solvation for the lesser polar resins **1** and **2** in terms of solvent polarity values (AN+DN) are located at <20 and ± 25, respectively (Fig. 1(A) and (B)). The presence of a greater number of polar peptide bonds in the peptide resins **3** and **4** shifted their areas of maximum solvation for solvents having polarity values of about 40–50 (Fig. 1(C) and (D)). These results confirm that polymeric materials achieve maximum solvation in solvents with polarities similar to that of their backbone,[3,4,8,27] thus revealing a clear relationship between peptide-resin solvation and polarity of the medium.

As discussed above, the solvents 21 and 22 (open circles) are composed of a strong electron acceptor (TFE) and strong electron donors (DMF and DMSO, respectively), and these components tend to interact with each other rather than disrupt closely associated peptide chains throughout the resin network (Table 1). As a consequence, the swellings observed for these two mixed systems were less extensive than what had been expected based upon their polarity values (Fig. 1(C) and (D)). Accordingly, this effect was even less pronounced when the solutes under study were peptide-free polymers (Fig. 1(A) and (B)).

In agreement with our initial presupposition of a self-neutralizing effect occurring in some single solvents when their electrophilicity and nucleophilicity powers are comparable, a clear lack of swelling was observed for MeCN, acetone and *i*PrOH (open circles 29–31, respectively), mainly towards peptide resins **3** and **4**. Interestingly, even in the peptide-free polymers **1** and **2**, MeCN and acetone were unable to solvate the resin matrices completely. In conclusion, these single solvents seem to typify organic solvents with a very weak solute solvation capacity, which is induced by an internal self-neutralizing effect in terms of electron acceptor/electron donor capacities. These findings confirm that the use of the electrophilicity and nucleophilicity terms is advantageous in interpreting solvent effect upon polymer-type solutes. None of the solvation data found for mixed 21 and 22 or for single 29–31 solvents could be explained if, for instance, other classical one-component polarity scales, such as Hildebrand's δ parameter,[28] Dimroth–Reichardt's E_T30[29] or even the classical dielectric constant ε,[30] were used in this approach.

Of note is that the present results emphasize the low resin-swelling capacity of MeCN, acetone and *i*PrOH and point

Table 1. Solvent parameters and swelling degrees of resins

Entry	Solvent	AN[a]	DN[a]	(AN+DN)	Resin[b]			
					1	2	3	4
1	Toluene	3.3	0.1	3.4	87	64	26	40
2	DCM	20.4	1.0	21.4	84	79	46	52
3	Chloroform	23.1	4.0	27.1	83	83	53	64
4	NMP	13.3	27.3	40.6	67	75	70	64
5	DMF	16.0	26.6	42.6	70	70	75	57
6	DMSO	19.3	29.8	49.1	51	71	76	65
7	TFE	53.5	0.0	53.5	28	77	63	60
8	EtOH	37.1	32.0	69.1	19	53	38	40
9	MeOH	41.3	30.0	71.3	17	59	45	41
10	Formamide	39.8	24.0	63.8	23	61	61	46
11	50% TFE/Toluene	28.4	0.1	28.5	71	82	62	64
12	20% TFE/DCM	27.0	0.8	27.5	72	78	70	60
13	50% TFE/DCM	36.9	0.5	37.5	56	80	73	58
14	80% TFE/DCM	46.9	0.2	47.4	42	80	75	65
15	20% DMSO/NMP	14.5	27.8	42.3	73	71	65	61
16	50% DMSO/THF	13.7	24.9	38.6	65	68	62	55
17	65% NMP/THF	11.5	24.8	36.1	79	75	68	66
18	50% DCM/DMF	18.2	13.8	32.0	70	76	66	61
19	50% DCM/DMSO	19.9	15.4	35.3	68	69	68	65
20	50% MeOH/DMSO	30.3	29.9	60.2	25	66	72	56
21	50% TFE/DMF	34.8	13.3	48.1	27	69	29	47
22	50% TFE/DMSO	36.4	14.9	51.3	28	70	31	47
23	10% TEA/DCM	18.5	6.6	25.1	76	81	60	62
24	10% TEA/DMF	14.5	30.0	44.5	66	78	69	65
25	10% TEA/DMSO	17.5	32.9	50.4	47	72	71	64
26	20% PIP/DCM	16.3	8.8	25.1	78	76	55	nd
27	20% PIP/DMF	12.8	29.3	42.1	73	75	66	nd
28	20% PIP/DMSO	15.4	31.8	47.2	62	71	70	nd
29	Acetonitrile	18.9	14.1	33.0	32	65	24	36
30	Acetone	12.5	17.0	29.5	48	63	21	40
31	2-Propanol	33.5	36.0	69.5	14	46	10	37

nd = not determined.
[a] Reference.[4,7]
[b] [(Swollen volume–dry volume)/swollen volume]×100 using the following values for measured diameters of dry beads: resins: **1** = 50 μm; **2** = 114 μm; **3** = 87 μm; **4** = 94 μm.

out to the need for caution in the application of some types of column chromatography in which these solvents are routinely used as a mobile phase.

2.2. Peptide solubilization

Paralleling previous correlation studies comparing peptide-resin solvation and polarity of the medium, solubility of four model peptide sequences was determined in 18 single or mixed solvents (Table 2). Differing from the resin solvation approach, water (single or mixed) was deliberately involved, together with other solvent systems previously applied in the evaluation of insolubility problems related to peptides and other macromolecules. The peptides coded **A** and **B** are those attached to resins **3** ($[NANP]_3$-Nle) and **4** (VHHQKLVFFAEDV), respectively. They were cleaved from the resin, purified conventionally by HPLC until homogeneous. The vasoactive bradykinin (RPPGFSPFR, BK) and the hydrophobic VVLGAAIV-amide segment[31] corresponding to the 291–298 fragment of the murine H-2K protein[32] were also introduced in this investigation as peptides **C** and **D**, respectively. Experimentally, a 10 mg/mL solution of each peptide was centrifuged for 1 h at 14,000 rpm and the supernatant and the precipitate were lyophilized until a constant weight was attained. In addition to values of the percentages of solubilization of each peptide, Table 2 also displays the AN, DN, (AN+DN) and (AN−DN) parameters of most of the solvent systems used in this study.

Differing from what was observed with peptide resins, no clear correlation was observed between degree of solubilization and polarity of solvent represented by the (AN+DN) or by any other scales (Fig. 2). This lack of correlation was also observed when the hydrophobicity of each sequence (values of 8.2, 45.7, 27.8 and 37.5 for peptides **A**–**D**, respectively), calculated from their amino-acid composition as previously reported,[33] was plotted against the polarity of the solvents (figure not shown). These findings stress the difference, in terms of dependence upon the polarity of the medium, between peptide chains that are free in solution and those that are bound to a polystructural network. The fundamental aspect distinguishing these two situations is the fact that, when attached to a polymer backbone, the overall degree of freedom, as well as the intra- or interchain association propensity of peptide chains, is, perforce, affected by the nature and structure of the neighboring polymeric environment. Conversely, in the case of peptide chains free in solution, they are characterized by having a higher range of mobility and a complex set of structural characteristics that influence the type and intensity of their intra- or interchain associations.

Despite the lack of an acceptable relationship with the polarity of the medium, the solubilization factor of peptides

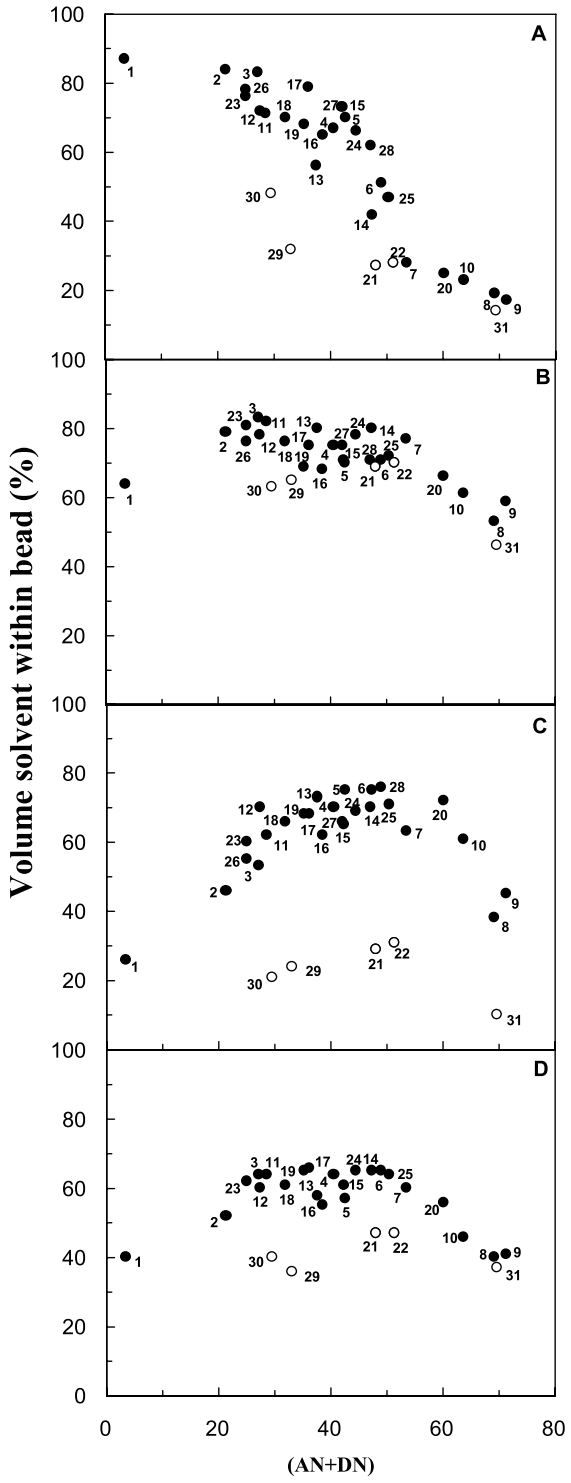

Figure 1. Swelling of resins (**1**), BHAR, 0.3 mmol/g [**A**], (**2**), PAC-PEG-PS, 0.18 mmol/g [**B**], (**3**), $(NANP)_3$-Nle-MBHAR, 2.6 mmol/g [**C**] and (**4**), VHHQKLVFFAEDV-MBHAR, 2.6 mmol/g [**D**] as a function of solvent polarity (AN+DN) values.

showed some correlation with the Lewis acid and Lewis basic properties of the medium, as represented by the AN and DN terms, respectively. In this context, the expected complementary participation of other factors, mainly the van der Waals forces, seems to be crucial for better evaluating the solubilization factor of each peptide sequence.

2.3. Effect of self-neutralizing (or heterogeneous) solvents

Initially, the results shown in Table 2 demonstrated that, in close agreement with their low capacity for disruption of peptide chains when bound to resins (low bead solvation), those single solvents denoted self-neutralizing, such as MeCN, acetone and *i*PrOH, also failed to dissolve peptides in solution, regardless of the sequence. However, their solubilization capacity changed profoundly when they were mixed with water, a strong electrophilic (or hydrogen bonding donor) solvent, characterized by an AN number of 54.8 (Table 2). Except for the strong aggregating peptide **D**, where the addition of water did not significantly increase their solubilization properties, the peptides were almost entirely dissolved in MeCN, acetone and *i*PrOH when cosolvated with 50% (v/v) water. These results may be attributable to the increased difference between AN and DN values (higher electrophilicity) in the mixed solvents resulting from the presence of water molecules (Table 2, AN−DN term). This may therefore favor the interaction of aqueous mixed solvents over that of single solvents, in which the difference in this physico-chemical parameter is quite low.

This unique effect of the water molecule is entirely governed by its overall hydrogen bonding property. However, due to its simple structure, it is, for instance, unable to promote the number of van der Waals (hydrophobic) interactions necessary for disruption of strongly aggregated chains, as can be seen for peptide **D** in water (Table 2). In this context, the further interpretation of solubilization data from more structured segments will be of great relevance in elucidating some rules that may control solubilization of peptides and macromolecules in general.

Many authors[34,35] have made mention of the tendency of these self-neutralizing solvents (mainly MeCN) to induce β-sheet strands rather than disordered or α-helix-type conformations in most peptide sequences, usually leading to aggregated states. However, none of these reports interpreted these findings in the light of the AN and DN concepts as detailed herein.

Relevant again for column chromatographic application, MeOH presented much higher peptide solubility power than did MeCN or *i*PrOH, both typifying organic solvents often applied in HPLC studies. Although this solubilization property increased proportionately with increased water in the mixture, it must be remembered that, as previously stated, these two weak solvating agents (MeCN and *i*PrOH) presented poor polymer solvation capability. This underscores the need for caution when these solvent systems are to be used in such experiments.

2.4. Effect of strong electrophilic or nucleophilic solvents

In general, only solvents comprising the strong electron donor DMSO (DN of 29.8) or the strong electron acceptor hexafluoroisopropanol (HFIP; AN of 88.0)[36] seemed able to completely dissolve aggregation sequences such as peptide **D**. In this case, solubilization percentages of 84 and 80%, respectively, were achieved. In contrast, the less

Table 2. Solvent parameters and solubility of individual peptides

Solvent	Parameter				Solubility of Peptide (%)			
	AN[a]	DN[a]	(AN+DN)	(AN-DN)	A	B	C	D
1. H_2O pH 3.0 and 9.0	54.8	18.0	72.8	36.8	100	100	100	0
2. MeCN	18.9	14.1	33.0	4.8	0	8	0	0
3. 50% MeCN/H_2O	36.9	16.1	53.0	20.8	100	76	100	0
4. Acetone	12.5	17.0	29.5	-4.5	0	0	0	0
5. 50% Acetone/H_2O	33.7	17.5	51.2	16.2	100	84	100	11
6. iPrOH	33.5	36.0	69.5	-2.5	0	0	0	10
7. 50% iPrOH/H_2O	44.2	27.0	71.2	17.2	100	100	88	25
8. MeOH	41.3	30.0	71.3	11.3	100	100	92	33
9. 50% MeOH/H_2O	48.1	24.0	72.1	24.1	100	100	88	26
10. TFE	53.5	0.0	53.5	53.5	100	100	92	20
11. 50% TFE/H_2O	54.2	9.0	63.2	45.2	100	100	100	60
12. HFIP	88.0	0.0	88.0	88.0	100	100	100	80
13. 50% HFIP/H_2O	71.4	9.0	80.4	62.4	100	100	100	70
14. DMSO	19.3	29.8	49.1	-10.5	100	100	100	84
15. 50% DMSO/H_2O	37.1	23.9	60.9	13.2	100	100	100	32
16. 3.0 M Urea	nd	nd	nd	nd	100	100	100	0
17. 6.0 M Urea	nd	nd	nd	nd	100	80	96	0

nd = not determined.
[a] Reference.[4,7]

electrophilic TFE (AN of 53.5) displayed much lower solubilization power in comparison with HFIP (20%). This significant difference in solubilization capacity between TFE and HFIP seems to be clearly due to the difference in their electrophilicity (AN increased from 53.5 to 88.0, respectively). However, to the strength of the van der Waals interaction induced by the presence of a second $-CF_3$ group in the HFIP structure is equally relevant. The appropriate combination of both effects (hydrogen bonding and hydrophobic or van der Waals forces) seems to play a crucial role in the significant increase in the disruption power of aggregated peptides, which is more pronounced when in HFIP.

The effect of the addition of water to single solvents is very complex but of great relevancy, as observed in the case of

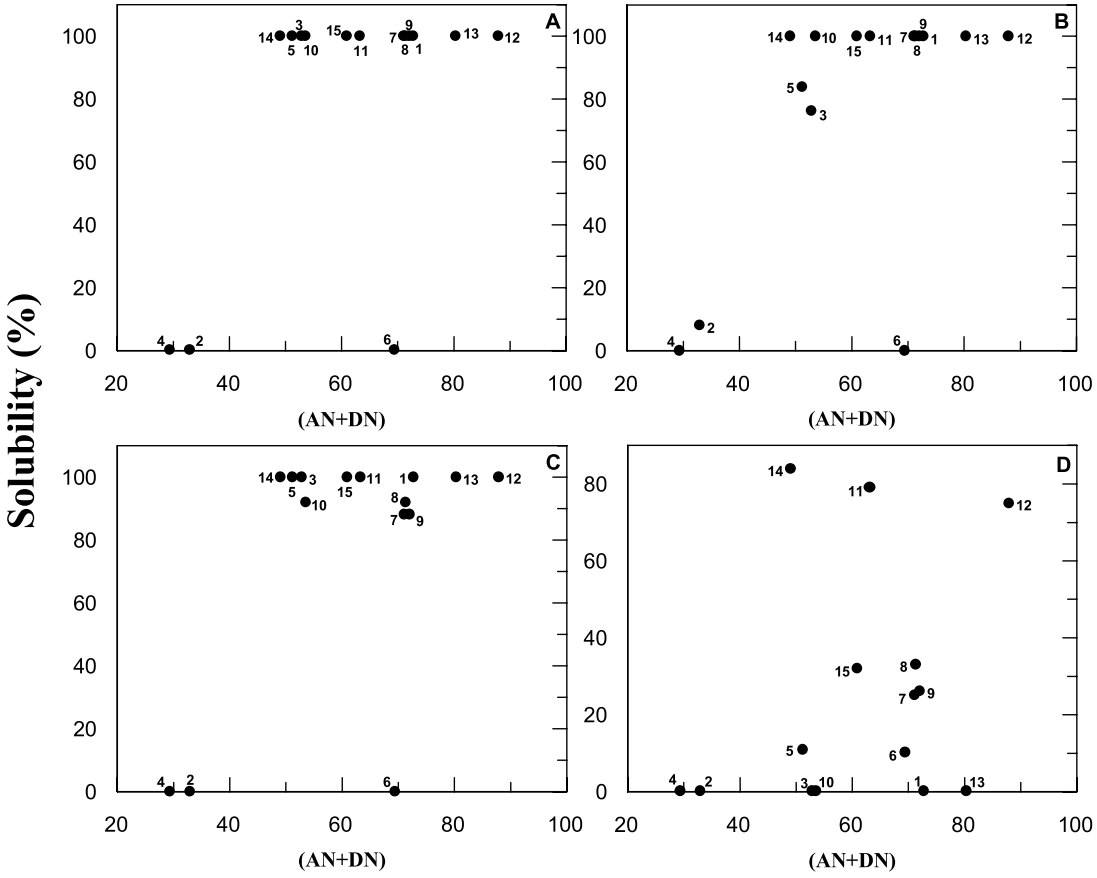

Figure 2. Solubility of peptides (**A**), $(NANP)_3$-Nle-amide, (**B**), VHHQKLVFFAEDV-amide, (**C**), RPPGFSPFR and (**D**), VVLGAAIV-amide as a function of solvent polarity (AN+DN) values.

the 'poor' solvents discussed in the previous section. When water was added to the strong polar organic solvents DMSO or HFIP, the solubilization yield decreased from 84 to 32% and from 80 to 70%, respectively (Table 2). These results indicate that the effect of the addition of water seems to be highly dependent upon the type of organic solvent to be mixed (electron acceptor or electron donor) and also on the particular characteristic of peptide sequence (degree of aggregation). When added to DMSO, the nucleophilicity of this polar aprotic solvent are partially neutralized by the strong electrophilicity of the water molecule, thus partially reducing the solubilization properties of the DMSO/water mixture. This effect is quite similar to the previously discussed solvation behavior of the heterogeneous mixed solvents 21 and 22, which are composed of strongly electrophilic and nucleophilic components (Table 1).

In contrast, when electrophilic water is added to other strong electrophilic solvents such as TFE or HFIP, a homogeneous solution is formed and the degree of alteration in their peptide chain dissolution potential seems very complex. For instance, in the case of HFIP, the addition of water slightly reduced the degree of peptide **D** solubilization (from 80 to 70%, respectively), whereas more significant variation (increases) in solubilization occurred in pure TFE or in TFE/water mixture (20 and 60%, respectively). Again, this result shows that the more pronounced effect seen when water was added to TFE than when it was added to HFIP could be credited to the much stronger electrophilicity of the latter fluorinated solvent, in combination with a higher potential to produce van der Waals interaction with aggregated peptide **D**.

In the literature, a great number of studies have examined peptide or other macromolecule solubility with the aim of finding rules that govern the effects of solvents with weak or strong dissociations.[18,34–38] In addition to these efforts, no clear explanation has been proffered for the apparently random way in solvents are currently chosen. For example, why would one consider opposite DMSO solvents to be more suitable than HFIP/TFE solvents? We have presented an alternative and, in some cases, consistent approach to address this extremely complex issue of solvent effects upon solute molecules. This approach relied mainly on the conjugated use of AN and DN terms (sum or difference), as well as on the potential for hydrophobic interaction, of all solvents involved in the interaction process, and on the specific characteristics of the peptide or peptide-resin solute components.

Due to the great complexity of the solute–solvent interaction, especially in cases involving peptide or peptide–polymer solutes, many further studies are warranted. In an attempt to depict this complexity, we have also included, in Table 2, dissolution data obtained when we used aqueous urea solutions, which are often proposed for use in the dissolution of proteins and peptides. A failure of such solutions to dissolve peptide **D** was observed, as well as, notably, an inverse relationship between solubility and urea concentration in peptide **B** dissolution. Otherwise, no correlation between solubility and media pH was observed, suggesting the absence of an ionization effect in some subgroups of the four peptides evaluated herein. Much larger numbers of solvent systems and solute models are currently under investigation in an attempt to further establish rules that might not only facilitate selection of the most suitable solvent for peptide dissolution or peptide-resin solvation but also be extended to many other solute–solvent interactions.

3. Conclusions

Despite the huge amount of data already existing in the literature, the solute–solvent interaction effect has eluded the scientific community for many decades. In a recent investigation, we combined the electron acceptor (AN) and electron donor (DN) parameters in order to build an alternative solvent polarity scale. As a continuation of this, we have, in the present study, evaluated these same physicochemical properties in order to interpret the complex dissociation process of peptide chains, comparing those free in solution with those coupled to polymers.

After investigating model peptides and peptide-resins solvated in a large number of solvent systems, we have reached several conclusions. First, in contrast to improved solvation of peptide-resins in solvents with similar polarities, the solubilization yield of a peptide in solution is not always directly related to the polarity of the medium. Second, optimal solubilization of peptides is strongly dependent upon the difference between AN and DN values of the solvent and of its ability to induce van der Waals attraction. In addition, mixed solvents with rather equivalent electrophilicity and nucleophilicity are not able to solvate aggregated peptide-resins or dissolve peptide sequences. This rule is also applicable to single solvents that present similar AN and DN values and induce a molecular self-neutralizing effect, thereby precluding dissociation of peptides in solution or solvation of peptide-resins. Furthermore, this self-neutralizing effect occurring in mixed or single solvents must be also considered for other biotechnological applications (such as in column chromatography) since it may affect solute solubilization and resin solvation simultaneously. Moreover, whether the addition of the strongly electrophilic water molecule to a mixture for peptide solubilization will be advantageous or not is clearly dependent on the relationship between acidity and basicity of both components. Finally, the peptide solubilization effect of urea in the solution is sequence dependent and, in some cases, involves an inverse correlation between solubility and urea concentration. Therefore, in light of the Lewis acidity and Lewis basicity properties of solvent systems, some relevant rules could be established for the complex processes of peptide dissolution and peptide-resin solvation.

4. Experimental

All amino-acid derivatives were purchased from Bachem (Torrance, CA, USA). Solvents and reagents were purchased from Fluka (Buchs, Switzerland), Aldrich-Sigma (Steinheim, Germany) and Advanced Chemtech (Louisville, KY, USA). The PAC-PEG-PS resin was acquired from Millipore (Bedford, CA, USA) and batches

of BHAR or MBHAR (0.3 and 2.6 mmol/g, respectively) were synthesized in our laboratory, following guidelines laid out in previous reports.[19,26]

4.1. Peptide synthesis

The peptides were synthesized manually according to the standard Boc[8] protocol. The following Boc amino-acid derivatives were used: Boc-Glu(OcHex), Boc-Asp(OcHex), Boc-Lys(2-Cl-Z), Boc-Ser(Bzl) and Boc-His(Tos). In the Boc chemistry, after coupling the C-terminal amino acid to the resin, the successive α-amino group deprotection and neutralization steps were performed in 30% TFA/DCM (30 min) and 10% DIEA/DCM (10 min). The amino acids were coupled using DIC/HOBt in DMF and, if necessary, TBTU in the presence of HOBt and DIEA using 20% DMSO/NMP as a solvent system. After a 2 h coupling period, the qualitative ninhydrin test was performed to estimate the completeness of the reaction. To check the purity of the synthesized peptide sequence attached to the resin, cleavage reactions with small aliquots of resin were carried out with the low-high HF procedure. Analytical HPLC, as well as LC/MS (electrospray) mass spectrometry (Micromass, Manchester, UK) and amino-acid analysis (Biochrom 20 Plus, Amersham Biosciences, Uppsala, Sweden), were used to check the homogeneity of each synthesized resin-bound peptide sequence.

4.2. Analytical HPLC

Analysis was performed in a system consisting of two model 510 HPLC pumps (Waters, Milford, MA, USA), an automated gradient controller, Rheodyne manual injector, 486 detector and 746 data module. Unless otherwise stated, peptides were analyzed on a 4.6×150 mm^2 column with a 300 Å pore size and a 5 μm particle size (C18; Vydac, Hesperia, CA, USA) using the solvent systems: A (H_2O containing 0.1% TFA) and B (60% MeCN in H_2O containing 0.1% TFA). A linear gradient of 10–90% B in 30 min was applied at a flow rate of 1.5 mL/min and detection at 220 nm.

4.3. Preparative HPLC

Purification of peptides was carried out using solvent A (H_2O containing 0.1% TFA) or solvent B (90% MeCN in H_2O containing 0.1% TFA). A linear gradient was applied which was dependent upon the retention time determined in the HPLC analysis of the peptide, using the same solvent systems. The flow rate was of 10 ml/min and the detection of peaks was carried out at 220 nm.

The following peptides deemed requisite for solubilization experiments were synthesized through Boc strategy:

(a) $(NANP)_3$-Nle-amide: this peptide was synthesized at 0.53 mmol scale starting from MBHAR resin (2.63 mmol/g). The crude peptide yielded 250 mg and, after HPLC purification, 165 mg of pure compound were obtained. ESI-MS, *m*/*z*: 1319 (theoretical), 1320 (obtained).

(b) VHHQKLVFFAEDV-amide: this peptide was synthesized in MBHAR (2.63 mmol/g) at 0.53 mmol scale. A total of 424 mg of crude peptide were obtained and, after HPLC purification, 104 mg of pure compound remained. ESI-MS, *m*/*z*: 1569 (theoretical); obtained (1569.2).

(c) VVLGAAIV-amide: this peptide was also synthesized in MBHAR (2.63 mmol/g) at 0.53 mmol scale After cleavage with HF procedure, 452 mg of crude peptide were obtained which yielded 126 mg after HPLC purification. ESI-MS, *m*/*z*: 740 (theoretical); 740.4 (obtained).

(d) RPPGFSPFR (BK): this peptide was synthesized in Boc-Arg(Tos)-PAM resin (0.6 mmol/g) at 0.5 mmol scale. After cleavage with HF procedure, 433 mg of crude peptide were obtained which yielded 273.4 mg after HPLC purification. ESI-MS, *m*/*z*: 1060 (theoretical); 1059 (obtained).

4.4. Measurements of bead swellings

Before use in peptide synthesis or microscopic measurement of bead sizes, most resin batches were sized by sifting through metal sieves to lower the standard deviation of resin diameters to about 4%. Swelling studies of these narrowly sized populations of beads have been previously conducted.[3] In short, 150–200 dry and swollen beads of each resin, allowed to solvate overnight, were spread over a microscope slide and measured directly with an Olympus model SZ11 microscope coupled with Image-Pro Plus version 3.0.01.00 software. Since the sizes in a sample of beads are log-normally rather than normally distributed, the more accurate geometric mean values and geometric standard deviations were used to estimate the central value and the distribution of the particle diameters. The resins were measured with their amino groups in the deprotonated form, obtained by 3×5 min washes in TEA/DCM/DMF (1:4.5:4.5, v/v/v), followed by 5×2 min washes in DCM/DMF (1:1, v/v) and 5×2 min DCM washings. Resins were dried in vacuum using an Abderhalden-type apparatus with MeOH reflux.

4.5. Solubility measurement of peptides

The solubility of each peptide was determined by dissolving 2.5 mg of pre-purified peptide in 0.25 mL (ca. 10 mM) of each of the solvents described in Table 2. The solution was centrifuged for 1 h at 14,000 rpm and the supernatant and the precipitate were lyophilized until constant weight was attained. Solubility data are expressed as percentages.

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