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Characterizing *N*-acetylcysteine (NAC) and *N*-acetylcysteine Amide (NACA) Binding for Lead Poisoning Treatment

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Abstract

Using antioxidants is an important means of treating lead poisoning. Prior *in vivo* studies showed marked differences between various chelator antioxidants in their ability to decrease both blood Pb(II) levels and oxidative stress resulting from lead poisoning. The comparative abilities of NAC and NACA to Pb(II) were studied *in vitro*, for the first time, to examine the role of the –OH/–NH₂ functional group in antioxidant binding behavior. To assay the antioxidant-divalent metal interaction, the antioxidants were probed as solid surfaces, adsorbing Pb(II) onto them. Surface characterization was carried out using X-ray photoelectron spectroscopy (XPS) analysis to quantify Pb(II) in the resulting adducts. XPS of the Pb 4f orbitals showed that more Pb(II) was chemically bound to NACA than NAC. In addition, the antioxidant surfaces were probed via point-of-zero charge (PZC) measurements of NAC and NACA were obtained to gain further insight into the Pb-NAC and Pb-NACA binding, showing that Coulombic interactions played a partial role facilitating complex formation. The data correlated well with solution analysis of metal-ligand complexation. UV-vis spectroscopy was used to probe complexation behavior. NACA was found to have the higher binding affinity as shown by free Pb(II) available in solution after complexation from HPLC data. Electrospray ionization mass spectrometry (ESI-MS) was applied to delineate the structures of Pb-antioxidant complexes. Experimental results were further supported by density functional theory (DFT) calculations of supermolecular interaction energies (E_{inter}) showing a greater interaction of Pb(II) with NACA than NAC.

Keywords

Lead poisoning; *N*-acetylcysteine; *N*-acetylcysteine amide; isoelectric point; density functional theory

INTRODUCTION

Lead poisoning is an ongoing world-wide concern. Lead accumulates in tissue [1] and has considerable adverse effects [2]. Lead, as a contaminant, emanates from a variety of sources, but mostly from drinking water [3]. The prevailing theory to explain the mechanism for lead

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toxicity is that Pb(II) oxidizes glutathione (GSH), resulting in the increase of free radicals. Since Pb(II) has a strong binding capacity for sulfhydryl proteins and enzymes, it will react with GSH to reduce the GSH-to-glutathione disulfide (GSSG) ratio, thereby causing GSH to lose its antioxidant abilities; instead, it functions as a scavenging reactive oxygen species (ROS) [4]. Chelation therapy using antioxidants is aimed at removing the accumulated lead in the body.

N-acetyl cysteine (NAC) and N-acetyl cysteine amide (NACA) have been touted as promising therapeutic antioxidant chelator agents [5,6]. The thiol and hydroxyl (NAC) and thiol and amine (NACA) groups within these respective antioxidant molecules are known to serve as chelation sites to which divalent metal ions coordinate. The acetyl group, common to both molecules, has additional antioxidant potential due for the generation of glutathione [7]. NAC, as an antioxidant, has widely been investigated for use in treating an array of diseases, including liver failure [8–10], inflammation [11], nephropathy [12] and brain disorders [7,13–15]. NAC's effectiveness is primarily attributed to its ability to reduce extracellular cystine to cysteine, and as a source of sulfhydryl groups [16]. NAC stimulates glutathione synthesis, enhances glutathione-S-transferase activity, promotes liver detoxification by inhibiting xenobiotic biotransformation, and is a powerful nucleophile capable of scavenging free radicals. Its use, however, has been limited by several drawbacks, including low membrane penetration and low systemic bioavailability. However, NACA, the amide form of NAC, the carboxyl group is neutralized which makes it more hydrophobic and membrane permeable. In fact, NAC is so hydrophilic that it is not able to cross blood brain barrier. NACA, the amide form of NAC, on the other hand, has a higher cellular permeability than NAC, and therefore is considered to be a noteworthy alternative therapeutic agent [16]. For this reason, NACA has been used to treat an array oxidative stress related diseases, for example the treatment of retinal degeneration and cataract formation [17,18], oxidative stress [19], and inflammatory lung injury [20].

Despite the interest in applying these molecules for therapeutic treatment, no work to date has been done to characterize the comparative binding behavior of these molecules to divalent Pb(II). Very little work has been done to characterize Pb-NAC binding [21], and none whatsoever has been done to characterize Pb-NACA binding. The difficulty in assaying the complexation to obtain binding constants stem from the fact that there are multiple species present when applying Job's method, hampering solution phase analysis. This phenomenon was observed in our own experimental results (*vide infra*). Therefore, we have taken a surface assaying approach using photoelectron spectroscopy to quantify the amount of Pb(II) bound to the antioxidants. The thiol and hydroxyl (NAC) and thiol and amine (NACA) groups within these respective antioxidant molecules are known to serve as chelation sites to which divalent metal ions coordinate. The acetyl group, common to both molecules, has additional antioxidant potential due for the generation of glutathione [22]. The NAC and NACA molecules are ideal for comparative study since they differ in structure only by the $-\text{NH}_2/-\text{OH}$ functional group. Hence, differences in overall Pb(II) binding to the ligands are attributable to these groups (Figure 1) within the ligand structures. The relative abilities of NAC and NACA to coordinate aqueous Pb(II) were examined by probing these materials as solid surfaces to gain insight into influence of $-\text{NH}_2/-\text{OH}$ group for binding, followed by solution complexation analysis.

EXPERIMENTAL

To assay the antioxidant-divalent metal interaction, NAC and NACA were probed as solid surfaces, adsorbing Pb(II) on them, respectively, followed by XPS analysis to quantify adsorbed Pb(II) in the adducts formed. Adsorption of Pb(II) from aqueous solution was performed onto NAC and NACA were performed, followed by electron spectroscopy.

(Detailed descriptions for the preparation of NAC and NACA surfaces for X-ray photoelectron spectroscopy and point-of-zero charge measurements, as well as instrumentation for solution complexation analysis and theoretical calculations are included in the Supporting Information section.) Surface characterization was complemented by solution complexation analysis. EDTA and cysteamine hydrochloride were used as standards for the Job's method using UV-vis spectroscopy and HPLC experiments, respectively, for comparative studies between NAC and NACA. Stock solutions of lead acetate and antioxidants of the same concentrations were prepared and maintained at a temperature between 0–10°C to prevent antioxidant decomposition just prior to analysis. The volume ratio of Pb(II) and antioxidants were varied from 7:1 to 1:3, according to Job's method [23]. When the volume ratio of two molecules is equal to their stoichiometric ratio, the amount of the complex formed would appear as a maximum in the UV-vis spectrum.

Point-of-zero charge (PZC) measurements of the NAC and NACA powders were conducted using the method described by Park and Regalbuto [24]. The PZC is defined as the aqueous solution pH value at which a solid surface has an electrostatically neutral charge. The antioxidant powders were used as the solid surface, incorporating them as supersaturated solutions, for the electrical double-layer model according to Gouy-Chapman theory.

The binding interactions of Pb(II) cation to NAC and NACA ligands were quantified using Density Functional Theory (DFT) [25] calculations using the Gaussian09 [26] suite of programs with Perdew-Wang 1991 exchange and correlation functionals [27,28] (Perdew, 1991; Perdew and Wang, 1992), a 6-311++G** basis set [29,30] for all ligand atoms, and a LANL2DZ basis set [31–33] for the Pb(II) cation, which uses effective core potentials (ECP) to describe core electrons. All calculations were performed using our own Linux Beowulf-type cluster equipped with 80×1.86 GHz Intel Xeon E5320 processors.

RESULTS AND DISCUSSION

XPS of the Pb 4f core levels were used to quantify the amount of complexed versus uncomplexed Pb(II) based on deconvoluted peak areas denoting these two populations. High resolution narrow scans of uncomplexed Pb acetate, deposited on an ultrahigh vacuum cleaned foil sample holder and outgassed via turbomolecular pump, showed oxidation states with binding energy peak centers at 138.4 (3.1) and 143.3 (2.8) eV for the 4f_{7/2} and 4f_{5/2} photoelectrons, respectively [with full-width at half maxima (fwhm) in parentheses]. In curvefitting the XPS of Pb-antioxidant complexes (Figure 1), these binding energy peak centers and fwhm denoting uncomplexed Pb(II) were constrained (dashed-line peak envelopes). The remaining peak envelopes were attributed to Pb(II) chemically bound to the antioxidants (dotted-line peak envelopes). The Pb 4f orbital was deconvoluted by four peak envelopes. Peaks denoted complexed Pb(II) for Pb-NAC had binding energies at 140.3 (3.0) and 146.2 (3.0) eV. For the Pb-NACA complex, these oxidation states were observed at 141.2 (3.2) and 145.6 (2.9) eV. The Pb 4f chemical environments attributed to complexed and uncomplexed Pb(II), were quantified using their respective XPS integrated peak areas. The sum of XPS peak areas from complexed (dotted-line envelopes) Pb(II) was divided by the total sum peak areas of both complexed and uncomplexed Pb(II).

Integrated peak areas of chemisorbed (dotted-line peaks in Figure 2) Pb 4f of high resolution scans, performed in triplicate, showed $11.6 \pm 2.6\%$, $11.2 \pm 3.5\%$ and $21.6 \pm 0.9\%$ Pb(II) chemically bound to the EDTA, NAC and NACA antioxidants, respectively. The percentage of complexed Pb(II), shown by XPS indicated that NACA had a higher binding affinity to Pb (II) than both NAC and EDTA. For Pb-EDTA, 11.6% of the Pb(II) was complexed. For Pb-NAC, 10.3% of the Pb(II) was complexed. For Pb-NACA, 21.6% of the Pb(II) was complexed, approximately twice of that for Pb-EDTA and Pb-NAC. The trend, showing a

greater degree of binding between Pb(II) with NACA than with NAC complements the HPLC data (*vide infra*).

To gain insight into the role of Coulombic interactions in the binding between Pb(II) and the antioxidants, the isoelectric points of NAC and NACA were measured, treating them as solid surfaces in aqueous solution environments. Figure 3 shows plots of initial versus final pH values for both NAC and NACA. The measured PZC values, denoted by the plateaus, were found to be at pH = 2.0 and pH = 5.1 for NAC and NACA, respectively. The solution pH (= 4.6) at which Pb-NAC and Pb-NACA complexations were performed was below the PZC of NACA, but higher than that of NAC. At the pH at which complexation of Pb(II) to the antioxidants occurred, NAC would adopt an overall negative charge and would be more amenable to bind Pb(II) while NACA would adopt an overall positive charge and repel Pb(II) according to the electrical double layer theory. Hence, the measured PZCs of NAC and NACA indicate that Coulombic interactions to the –OH/–NH₂ group only partially plays a role in governing binding between Pb(II) and the antioxidants. If this were the sole interaction, a greater binding between Pb and NAC than Pb and NACA would be predicted. The greater binding affinity of Pb(II) to NACA than to NAC, as observed by XPS and HPLC, is also incongruent with known electronegativities of the –OH and –NH₂ functional groups, which in NAC and NACA differ in structure. Since –OH is the more electronegative than –NH₂ based on the bond polarity index [34], a greater affinity of Pb(II) to NAC than Pb-NACA binding would have been predicted; however, both HPLC and XPS results showed the opposite trend. This finding indicates that it is the interplay of a variety of interatomic forces (not electrostatic ones only) between Pb(II) interacting with multiple atoms within the ligand that governs the binding strength of the antioxidant chelator molecule. This rationale is further supported by DFT calculations (*vide infra*).

The left hand panel of Figures 4A, B and C shows raw UV-vis absorbance data of aqueous Pb(II) complexing with antioxidants EDTA, NAC and NACA, respectively. Actual concentrations of the Pb(II) substrate and antioxidant ligand to form the complexes for analysis are summarized in Table 1. The right-hand panel shows Pb-antioxidant complex concentrations (in arbitrary units) as a function mole fraction, x , determined by Job's method analysis. The n/m ratio signifies the substrate-ligand complex, S_mL_n . The n and m variables that represent stoichiometric numbers for the ligand and substrate, respectively,

were determined by the relation, $\frac{n}{m} = \frac{x_{\max}}{1 - x_{\max}}$, in which $x_{\max}$ is the mole fraction of maximum complex concentration. Figure 4A shows UV-vis spectra Pb-Na₄EDTA complexation, showing a maximum absorbance at 246 nm. For the Pb-EDTA complex, $x_{\max} = 0.5$, denoting a 1:1 stoichiometric ratio in good agreement with the literature [21] and validating the accuracy of our approach. Figure 4B shows the results for Pb-NAC complexation. The maximum absorbance of these spectra was observed at 275 nm. With $x_{\max} = 0.286$, the stoichiometric ratio was 2.5:1 for Pb:NAC. Figure 4C show spectra for Pb-NACA complex formation. The wavelength of maximum absorption was the same as that for Pb-NAC complex formation, at 275 nm. With $x_{\max} = 0.400$, the ratio Pb-to-NACA in the complex was found to be 1.5:1. Non-unity stoichiometries for Pb-NAC and Pb-NACA denoted more than one complex being formed as Pb(II) interacted with the NAC and NACA antioxidants; hence, binding constant determination for these complexes were hampered. Substrate-to-ligand ratios in the complexes formed for Pb-NAC and Pb-NACA differed, giving further support that there is substantially dissimilar coordination behavior between Pb(II) and the two antioxidants.

To gain insight into the structures of these complexes, ESI-MS was performed to detect and characterize the Pb-antioxidant mass spectral (MS) cracking fragments. Major complexes examined were those of the Pb-EDTA (used as a standard), Pb-NAC and Pb-NACA

complexes. Figure 5A shows the signal emanating from $[\text{Na}_3\text{EDTA-Pb}]^+$ at 565.09 m/z, in agreement with the expected 1:1 ratio of the Pb-EDTA complex in the UV-vis experiments; this was the only predominant fragment observed [21]. Figure 5B shows the MS patterns of the Pb-NAC complex. Four MS fragments were detected, indicating that Pb(II) coordinated to NAC with metal-to-ligand ratios of 1:1, 2:1, 2:2, and 3:2. Masses were observed from $[\text{Pb-NAC}]^+$ at m/z = 369.77 m/z, $[\text{Pb-NAC-Pb-H}_2\text{O}]^+$ at m/z = 591.82, $[\text{NAC-Pb-NAC-Pb}]^+$ at m/z = 736.80, and $[\text{Pb-NAC-Pb-NAC-Pb}]^+$ at m/z = 942.73; the percent mole fractions for all of these species were 76.5%, 14.5%, 6.5%, and 2.5%, respectively. In contrast to the Pb-NAC binding, only three major MS fragments were observed in the Pb-NACA complexation according to ESI-MS (Figure 5C). One signal emanated from $[\text{Pb-NACA-H}]^+$ at m/z = 368.93. Two other peaks were observed at m/z = 529.08 and m/z = 575.04, denoting $[\text{Pb-(NACA-NACA)-H}]^+$ and $[\text{Pb-NACA-Pb}]^+$. Mole fraction percent compositions of $[\text{Pb-NACA-H}]^+$, $[\text{Pb-(NACA-NACA)-H}]^+$ and $[\text{Pb-NACA-Pb}]^+$ for Pb-NAC complexation were 92.2%, 4.8% and 3.0%, respectively. In the cases of both Pb-NAC and Pb-NACA coordination interactions, the 1:1 substrate-to-ligand ratio was the most dominant stoichiometry for Pb-antioxidant coordination. Multiple Pb atom species detected by ESI-MS correlates with the high Pb-to-antioxidant ratios observed in the Job's method data (Figures 4B and 4C). Although the Pb:antioxidant ratios could not be directly quantified from percent mass spectral abundances, the presence of higher Pb-to-ligand ratio fragments for Pb-NAC than Pb-NACA is consistent with observed n/m ratios in the Job's method experiments.

Cysteamine hydrochloride, NAC and NACA are mono-thiol antioxidants that contain a sulfur-hydrogen functional group. Chelating ability was determined and evaluated by quantifying the percentage of Pb(II) coordinated to the antioxidants, and HPLC was applied to determine the amount of antioxidants that had been coordinated. The retention times for NAC, NACA and cysteamine were 4.8, 5.2 and 17.2 min, respectively. Figure 6 shows the declining trend of cysteamine with an increase of Pb acetate concentration. With addition of Pb(II) acetate, the decrease in free cysteamine, NAC, and NACA in the substrate-ligand complexes became more pronounced (Table 2). At a 1:1 ratio of Pb:cysteamine, there was a 19.0% decrease in the free cysteamine concentration (compared to the control that contained only the ligand) with zero incubation time, and a 33.5% decline after a 1-hr incubation in 37.0°C water-bath. This decrease in free cysteamine is attributed to antioxidant decomposition at physiological temperature, which were also observed for NAC and NACA. At a 1:1 ratio of Pb-to-NAC, there was 10.1% decrease in free NAC in solution upon its interaction with Pb(II) at no incubation; after a 1-hr incubation, there was 15.5% decrease of free NAC. For the Pb-NACA complex, there was a 21.7% decrease in free NACA at zero incubation and a 28.7% decrease after a 1-hr incubation. HPLC data showed the relative amounts of free ligands complexing to Pb(II) in the following descending order: NAC > NACA > cysteamine. From the decrease in free antioxidant in solution, the data set indicated that a relatively greater proportion of NACA coordinated to Pb(II) as compared to NAC, i.e., NACA had a greater affinity for Pb(II) than NAC.

In the DFT calculations, approximately two dozen possible metal-ligand structures for each coordination were calculated, with inter-atomic distances between the Pb(II) atom and the nearest (1NN), second (2NN), third (3NN), fourth (4NN) and fifth (5NN) nearest neighbor atoms reported. The presence of the -OH/-NH₂ functional groups affected the optimized geometries of the divalent metal with ligands, and subsequently the overall Pb(II) interactions with multiple atoms within the antioxidant. There were a total of twenty four possible structures for NAC-Pb (Table S1) and twenty three possible structures for Pb-NACA (Tables S1 and S2, respectively, shown in the Supporting Information section). Interatomic distances between Pb(II) atom and the first five nearest neighbors are listed along with its intermolecular interaction binding energy (E_{inter}), the energy needed to

remove the metal atom from each cluster in the data tables. In addition to atomic level electrostatic interaction energies, the calculated stabilization energy denoted in E_{inter} includes contributions from dispersion, induction and exchange repulsion energies between the Pb(II) and its nearest neighbors, which are not fully accounted for by the relative electronegativities of the -OH/-NH_2 groups within the NAC and NACA molecules or isoelectric points of these ligands alone. The average E_{inter} for Pb-NAC and Pb-NACA were found to be -4.7 ± 1.2 eV and -5.1 ± 1.5 eV, respectively (Tables S1 and S2), confirming that Pb(II) has a greater binding affinity for NACA than NAC.

Questions regarding the PZC data not only provided motivation for the DFT calculations, they (Figure 2) also reveal an additional insight into a property of NAC and NACA, influenced by the -OH/-NH_2 functional group. The isoelectric point of NACA (PZC = 5.1) is closer to cytoplasm pH (= 7.2–7.4) than that of NAC (PZC = 2.0), hence making NACA more amenable to traverse cell membranes. Therefore, compared to NAC, NACA, in addition to having a greater binding affinity for Pb(II), may be the better ligand to treat lead poisoning due to a greater propensity to remove intracellular Pb(II) across membranes.

CONCLUSIONS

In summary, the solution complexation data correlated well with the surface analysis assays provided by XPS and isoelectric point measurements. UV-vis Job's method data indicated multiple complexes present as Pb(II) coordinated with the NAC and NACA, respectively, which was supported by ESI-MS data. Fragmentation patterns for Pb-NAC and Pb-NACA showed four complexes in the Pb-NAC sample with metal-to-ligand ratios of 1:1, 2:1, 2:2, and 3:2. For Pb-NACA, metal-to-ligand ratios of 1:1, 2:1 and 2:2 were observed. Among both metal-to-ligand coordinations, the 1:1 ratio was the most abundant as observed by ESI-MS. The presence of fewer complexes in the Pb-NACA coordination as compared to Pb-NAC suggests a greater stability complex formation for Pb(II) interactions with NACA. Both HPLC and XPS of the Pb 4f core levels showed a greater population of chemically bound Pb(II) to the antioxidants than unbound Pb(II), i.e., unreacted Pb(II), for Pb-NACA than Pb-NAC. PZC measurements of NAC (2.0) and NACA (5.1), indicated that electrostatic effects from the -OH/-NH_2 moieties alone (consistent with the known polarizabilities of these groups) only partially accounted for the greater Pb-NACA binding, compared to Pb-NAC. These *in vitro* findings in this study will serve as an important benchmark for engineering improved therapeutic antioxidant chelators for the treatment of lead poisoning. DFT calculations of the E_{inter} binding energies showed that the interplay of (1) electrostatic interactions, (2) dispersion, (3) induction and (4) exchange repulsion energies accompanying the optimized geometries of the metal-ligand structures governed the observed, enhanced Pb-NACA binding. Regarding the precise mechanism governing Pb-antioxidant binding, the precise role of each of these contributions for metal-ligand binding is under currently under investigation in a more rigorous theoretical calculation study. Our current findings suggest that the collective contributions of (2), (3) and (4) is more substantial as compared to (1) alone.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Structural formulas for (A) N-acetyl cysteine (NAC); and (B) N-acetyl cysteine amide (NACA). Numeric assignments for each atom shown correspond to atomic positions used for DFT calculations. Hydrogen atoms attached to C(7) were assigned as H(11) and H(12).

Figure 2. XPS Pb 4f spectra of (A) Pb-NAC; and (B) Pb-NACA. Dotted-line envelope denotes Pb(II) chemically bound to NAC while the dashed-lined envelope denoted uncomplexed Pb acetate.

Figure 3. PZC Plots for NAC and NACA. Plateaus denote the observed PZC for NAC and NACA at 2.0 and 5.1, respectively. The pH at 4.6 (horizontal dotted line) denotes aqueous solution conditions at which Pb-NAC and Pb-NACA complexations occurred.

Figure 4.

Raw UV-vis spectral data (left-hand panel) and Job's method plots (right-hand panel) Pb-antioxidant complexes. **(A)** The wavelength of maximum absorbance ($x_{\max}$) for Pb-EDTA was 246 nm with a metal-ligand stoichiometric ratio of 1:1; **(B)** the highest $x_{\max}$ for Pb-NAC was at 275 nm, with a 2.5:1 ratio; and **(C)** the $x_{\max}$ for Pb-NACA was at 275 nm with a 1.5:1 stoichiometric ratio.

Figure 5. ESI-MS cracking pattern of (a) Pb-EDTA solution; (b) Pb-NAC; and (c) Pb-NACA.

Figure 6.

HPLC data showing free antioxidants in solution with increasing Pb acetate concentrations added. The control group contained only the respective antioxidants at 12.5 nM concentration. Samples from which HPLC data were obtained were comprised of 12.5nM of antioxidant, with increasing Pb acetate concentrations of 6.25, 12.5, 25.0 and 50.0 nM, respectively. (A) Pb-NACA solutions; (B) Pb-NAC solutions; and (C) Pb-cysteamine hydrochloride solutions.

TABLE 1

Composition of Pb-antioxidant complexes obtained from ESI-MS

Ligand	Mass-to-charge ratio (m/z)	Fragment	Percent abundance
Na ₄ EDTA	565.09	[Na ₃ -EDTA-Pb] ⁺	100%
NAC	369.77	[Pb-NAC] ⁺	76.5%
	591.82	[Pb-NAC-Pb-H ₂ O] ⁺	14.5%
	736.80	[NAC-Pb-NAC-Pb] ⁺	6.5%
	942.73	[Pb-NAC-Pb-NAC-Pb] ⁺	2.5%
NACA	368.93	[Pb-NACA-H] ⁺	92.2%
	529.08	[Pb-(NACA-NACA)-H] ⁺	4.8%
	575.04	[Pb-NACA-Pb] ⁺	3.0%

TABLE 2

Percentage of Antioxidants Reacted with Pb(II) from HPLC

Ligand	Ligand:Pb(II) ratio							
	1:0.5		1:1		1:2		1:4	
	0h	1h	0h	1h	0h	1h	0h	1h
NACA	25.5%	22.9%	21.7%	28.7%	25.4%	36.1%	29.7%	46.1%
NAC	7.8%	16.0%	10.1%	15.5%	12.0%	17.4%	12.5%	23.3%
Cysteamine	18.5%	29.1%	19.0%	33.5%	20.7%	24.1%	23.7%	34.8%