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Silver Sub-nanoclusters Electrocatalyze Ethanol Oxidation and Provide Protection against Ethanol Toxicity in Cultured Mammalian Cells

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Abstract: Silver atomic quantum clusters (AgAQCs), with two or three silver atoms, show electrocatalytic activities that are not found in nanoparticles or in bulk silver. AgAQCs supported on glassy carbon electrodes oxidize ethanol and other alcohols in macroscopic electrochemical cells in acidic and basic media. This electrocatalysis occurs at very low potentials (from $\sim +200$ mV vs RHE), at physiological pH, and at ethanol concentrations that are found in alcoholic patients. When mammalian cells are co-exposed to ethanol and AgAQCs, alcohol-induced alterations such as rounded cell morphology, disorganization of the actin cytoskeleton, and activation of caspase-3 are all prevented. This cytoprotective effect of AgAQCs is also observed in primary cultures of newborn rat astrocytes exposed to ethanol, which is a cellular model of fetal alcohol syndrome. AgAQCs oxidize ethanol from the culture medium only when ethanol and AgAQCs are added to cells simultaneously, which suggests that cytoprotection by AgAQCs is provided by the ethanol electro-oxidation meditated by the combined action of AgAQCs and cells. Overall, these findings not only show that AgAQCs are efficient electrocatalysts at physiological pH and prevent ethanol toxicity in cultured mammalian cells, but also suggest that AgAQCs could be used to modify redox reactions and in this way promote or inhibit biological reactions.

1. Introduction

Electrochemistry mediated by small metal nanoparticles is a recent, very active and promising field with enormous possibilities, many of which remain unexplored.[1] Molecule-like energy HOMO−LUMO (the highest occupied molecular orbital to the lowest unoccupied molecular orbital) gaps are created in nanoparticles with <100 atoms, which are called atomic clusters. The existence of these band gaps together with geometrical or electronic closed-shell structures[2] confers novel properties to atomic clusters[3−6] as well as an unexpected extremely high stability.[7−9] For example, we have recently reported the fluorescent[10] and paramagnetic[11] properties of very stable Ag_n ($n < 10$) clusters and the excellent electrocatalytic properties shown by Au_n ($n \approx 3-11$) clusters for the oxygen reduction reaction.[12] As we will show later on, we also observe that silver atomic quantum clusters (AgAQCs) containing only 2 or 3 atoms are very active electrocatalysts in the oxidation of alcohols, and this occurs at very low potentials. On the basis of these properties, we hypothesized that electrocatalysis by AgAQCs for ethanol oxidation reaction is possible whenever cells provide the required electrical potential for the electrochemical oxidation reaction. In this case, AgAQCs could prevent (or at least mitigate) some cellular injuries produced by ethanol. This is relevant in the case of fetal alcohol syndrome (FAS), a common pathology in children of women who consumed alcohol

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(1) Murray, R. W. *Chem. Rev.* **2008**, *108*, 2688.
(2) Negishi, Y.; Chaki, N. K.; Shichibu, Y.; Whetten, R. L.; Tsukuda, T. *J. Am. Chem. Soc.* **2007**, *129*, 11322.
(3) Heaven, M. W.; Dass, A.; White, P. S.; Holt, K. M.; Murray, R. W. *J. Am. Chem. Soc.* **2008**, *130*, 3754.
(4) Negishi, Y.; Takasugi, Y.; Sato, S.; Yao, H.; Kimura, K.; Tsukuda, T. *J. Am. Chem. Soc.* **2004**, *126*, 6518.
(5) Schaeffer, N.; Tan, B.; Dickinson, C.; Rosseinsky, M. J.; Laromaine, A.; McComb, D. W.; Stevens, M. M.; Wang, Y.; Petit, L.; Barentin, C.; Spiller, D. G.; Cooper, A. I.; Levy, R. *Chem. Commun.* **2008**, *34*, 3986.
(6) Herzing, A. A.; Kiely, C. J.; Carley, A. F.; Hutchings, P. L. G. J. *Science* **2008**, *321*, 1331.
(7) Duan, H.; Nie, S. *J. Am. Chem. Soc.* **2007**, *129*, 2412.
(8) Li, J.; Li, X.; Zhai, H.-J.; Wang, L.-S. *Science* **2003**, *299*, 864.
(9) Shichibu, Y.; Negishi, Y.; Tsunoyama, H.; Kanehara, M.; Teranishi, T.; Tsukuda, T. *Small* **2007**, *3*, 835.
(10) Ledo, A.; Martínez, F.; López-Quintela, M. A.; Rivas, J. *Physica B* **2007**, *398*, 273.
(11) Ledo-Suarez, A.; Rivas, J.; Rodriguez-Abreu, C. F.; Rodriguez, M. J.; Pastor, E.; Hernandez-Creus, A.; Osseroff, S. B.; López-Quintela, M. A. *Angew. Chem., Int. Ed.* **2007**, *46*, 8823.
(12) Rodríguez-Vázquez, M. J.; Blanco, M. C.; Lourido, R.; Vazquez-Vazquez, C.; Pastor, E.; Planes, G. A.; Rivas, J.; López-Quintela, M. A. *Langmuir* **2008**, *24*, 12690.

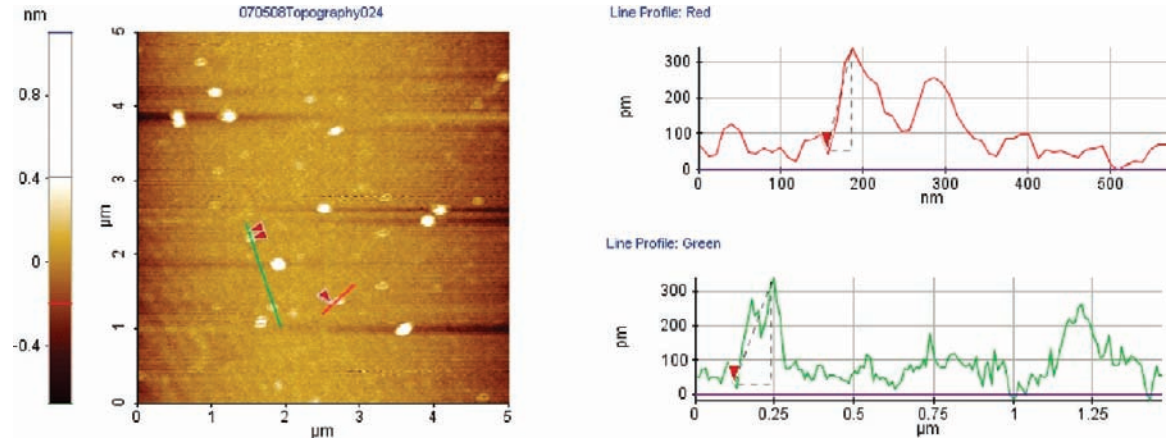

Figure 1. (Left) AFM picture of AgAQCs deposited on mica (mean square roughness ~150 pm). (Right) Profiles throughout the red and green lines depicted on the AFM picture.

during pregnancy.[13] It is important to highlight that the development of the nervous system is particularly sensitive to alcohol, which can finally lead to severe dysfunctions.[13] In this respect, it has been reported that ethanol induces, among other effects, apoptosis[14] and actin cytoskeleton rearrangements[15,16] in primary cultures of newborn rat astrocytes, which is a well-established cellular model of FAS.[13]

Here, we show that AgAQCs are cytoprotective against ethanol, which is revealed by the prevention of actin cytoskeleton rearrangements and apoptosis caused by ethanol exposure. We hypothesize that the possible correlation between the electrocatalytic and cytoprotective properties of AgAQCs follows the well-known role played by enzymes in fuel cell technologies. A great interest in the use of enzymes instead of metals as electrocatalysts in fuel cell reactions has recently emerged in this field.[17] However, to the best of our knowledge, the application of metals as electrocatalysts for changing biological reaction rates has not been explored so far. Our *in vivo* and *in vitro* experiments, showing that AgAQCs are effective electrocatalysts for electro-oxidation of different alcohols in both media, demonstrate that the connection between electrochemical and biological cells may be much deeper than it has been thought up until now.[17]

Thus, we provide experimental evidence that (1) AgAQCs supported on glassy carbon electrodes electrocatalyze the oxidation of ethanol in macroscopic electrochemical cells in acidic and basic media at voltages near 0 V vs the reference hydrogen electrode (RHE), at very low potentials in a physiological medium (PBS) and pH, and at ethanol concentrations found in alcoholic patients; (2) AgAQCs prevent ethanol-induced alterations in normal rat kidney (NRK) cells and primary cultures of newborn rat astrocytes; (3) AgAQCs reduce the ethanol concentration present in the culture medium of cultured cells; and (4) AgAQCs electro-oxidize and prevent the toxicity induced by other alcohols such as methanol and butanol, but not other stress-induced agents such as H_2O_2. Finally, we suggest a mechanism for electrocatalysis of the ethanol oxidation by AgAQCs in cultured mammalian cells.

2. Experimental Section

Antibodies and Reagents. Polyclonal anti-actin antibody was purchased from Cytoskeleton (Denver, CO). Polyclonal anti-caspase-3 and anti-cleaved caspase-3 antibodies were from Cell Signaling Technology (Danvers, MA). Monoclonal anti-α-tubulin antibody was from Sigma-Aldrich. Secondary goat anti-rabbit and goat anti-mouse IgG HRP antibodies were from Promega (Eugene, OR). Cyanine-3 (Cy3)-conjugated rabbit, Cy3-conjugated mouse, and Cy2-conjugated mouse secondary antibodies were from Jackson Laboratories (CSH, USA). Cell culture media were obtained from Gibco BRL Life Technologies (Paisley, Scotland, UK). Latrunculin B and DAPI were obtained from Calbiochem (Darmstadt, Germany). Ethanol, methanol, butanol, and H_2O_2 were from Merck (Nottingham, UK). Staurosporine, TRITC-phalloidin, FITC-phalloidin, yeast alcohol dehydrogenase, NAD^+, and the other chemical reagents were purchased from Sigma-Aldrich. All chemicals were of reagent grade, purchased from Fluka and used as received. Solutions were prepared with deionized water (Milli-Q, Millipore Corp). Silver clusters (NGAP-AQC-Ag01) dispersed in aqueous solution, mainly composed of Ag_2L and Ag_3L ($L = OH^-$, O_2, and/or H_2O) clusters at a concentration of 1 mg/L, were purchased from Nanogap Co. (Santiago de Compostela, Spain, www.nanogap.es). Ag clusters were used as provided, without further purification.

Other Experimental Procedures. See Supporting Information

3. Results and Discussion

3.1. AgAQCs Catalyze the Electro-oxidation of Ethanol at Very Low Potentials. We first checked the size and stability of AgAQCs. Atomic force microscopy (AFM) measurements of clusters deposited on a mica surface confirmed the presence of very small clusters ~0.3 nm high, i.e., 1 atom high (Figure 1). This meant that Ag clusters should contain less than ~6−7 atoms, because clusters of this size have a 2D flat structure.[18]

Ag clusters are very stable in water dispersions (for up to several months), displaying a continuous increase of spectral absorption in the UV region with decreasing wavelength and showing some resolved peaks (Figure S1, Supporting Information), which is characteristic of very small clusters.[12] An estimation of the cluster size assuming the spherical Jellium model (which seems to be acceptable even for very small

(13) Martínez, S. E.; Egea, G. *Recent Pat. CNS Drug Discovery* **2007**, *2*, 23.
(14) Miñambres, R.; Guasch, R. M.; Perez-Aragó, A.; Guerri, C. *J. Cell Sci.* **2006**, *119*, 271.
(15) Tomás, M.; Marín, P.; Megías, L.; Egea, G.; Renau-Piqueras, J. *Neurobiol. Dis.* **2005**, *20*, 773.
(16) Martínez, S. E.; Lázaro-Dieguez, F.; Selva, J.; Calvo, F.; Piqueras, J. R.; Crespo, P.; Claro, E.; Egea, G. *J. Neurochem.* **2007**, *102*, 1044.
(17) Cracknell, J. A.; Vincent, K. A.; Armstrong, F. A. *Chem. Rev.* **2008**, *108*, 2439.
(18) Lee, H. M.; Ge, M.; Sahu, B. R.; Tarakeshwar, P.; Kim, K. S. *J. Phys. Chem. B* **2003**, *107*, 9994.

clusters)[19] from the beginning of the absorption bands (~350 nm = 3.54 eV) gives a size of ~3 atoms ($n = (5.4/3.54)^3$, where 5.4 V is the Fermi level of bulk silver), which in turn is consistent with the data supplied by the supplier. We also confirmed that AgAQCs are very stable in the potential range from −0.8 to +0.7 V vs RHE (data not shown). The high stability of these small clusters is probably associated with their large HOMO−LUMO bandgaps (> ~3.5 eV), which renders them very difficult to reduce or oxidize, and also with their strong electronic shell closure ($1S^2$) predicted for delocalized "superatomic orbitals".[20]

Next, we examined the electrocatalytic properties of AgAQCs. To this aim, we tested the oxidation of ethanol. Linear sweep voltammograms (LSVs) from −200 to +300 mV vs RHE were recorded using a modified glassy carbon (GC) electrode containing silver clusters. This particular range of low potentials was chosen because this is the range of interest for the electrocatalytic applications of clusters in biological cells. The amount of clusters used was calculated to achieve approximately a monolayer of Ag clusters deposited onto the GC electrodes. LSVs were carried out first in a blank solution (acid medium, 1 M H_2SO_4, or basic medium, 1 M NaOH) and then in the same solution but containing different ethanol concentrations. Oxidation of ethanol occurred in both acidic and basic media (Figure 2A,B, respectively). AgAQCs required very low potentials to promote electrocatalysis (voltages close to 0 V vs RHE; Figure 2A). Similar results were obtained using electrodes covered with Nafion (data not shown), which indicates that AgAQCs are stable on the bare GC electrode surface within the explored potential range. The higher anodic currents detected in the basic solution are consistent with the observation that, in a basic medium, the adsorption of spectator anions on silver decreases, thus hindering ethanol oxidation (Figure 2B).[21]

Since the initial electrocatalytic results were obtained in strong acidic and basic media, we next examined whether AgAQCs are also able to oxidize ethanol in physiological conditions. For this purpose we used phosphate buffered saline (PBS; pH 7.4). Moreover, we sought to identify the range of current densities in which the electrocatalysis occurs in the oxidation of the ethanol at concentrations that are usually found in blood of alcoholic individuals.[22] Interestingly, the electro-oxidation of ethanol (at 20, 100, and 500 mM) in PBS took place at +0.2 V vs RHE (Figure 2C).

The nonlinear dependence on the current density of the ethanol concentration at these low potentials (< ~0.3 V) indicates that the electro-oxidation of ethanol does not occur under diffusive control but by the applied potential, which in turn is also supported by the voltammetric dependences on the scan rate (see Figure S2A,B, Supporting Information). Similar behavior is found in the glucose oxidation by enzymes attached to carbon electrodes under the same range of potential.[23] Moreover, the observed current densities ($\approx \mu A/cm^2$) for ethanol

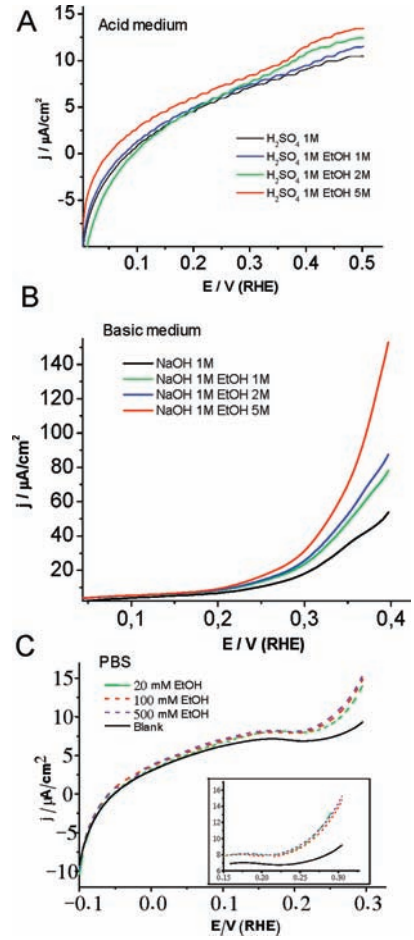

Figure 2. Electrocatalysis of the oxidation of ethanol by AgAQCs. Linear sweep voltammograms at different concentrations in acid (H_2SO_4, A), base (NaOH, B), or physiological medium (PBS, C). All potentials are referred to the reference hydrogen electrode (RHE). Sweep rate, 50 mV s^{-1}.

are of the same order of magnitude of those reported for electro-oxidation reactions carried out on enzymatically modified electrodes.[17] The reaction products and exact pathway by which ethanol electro-oxidation takes place with Ag clusters at these low overpotentials are at the moment not known, but the catalytic activity could be related to their ability to interact with oxygen-containing species.[24] The observed catalytic activity displayed by AgAQCs can be compared with that of other biological and nonbiological catalysts under similar experimental conditions. Thus, the estimated catalytic activity of AgAQCs in acidic medium and 0.5 V is 0.15 $mA/cm^2 \cdot mg$ of Ag. This catalytic activity is ~100 times higher than that observed for Pt, and half that of $Pt_{80}Ru_{20}$.[25] Comparison with other enzymes, such as alcohol dehydrogenase (ADH) and its co-enzyme nicotinamide adenine dinucleotide (NAD^+/NADH), is more difficult due to the wide dispersion of reported values. For example, the activity of AgAQCs at physiological pH and 0.2 V is approximately of the same order of magnitude as that

(19) Zheng, J.; Zhang, C.; Dickson, R. M. *Phys. Rev. Lett.* **2004**, *93*, 077402.
(20) Walter, M.; Akola, J.; Lopez-Acevedo, O.; Jadzinsky, P. D.; Calero, G.; Ackerson, C. J.; Whetten, R. L.; Grönbeck, H.; Häkkinen, H. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 9161.
(21) Spendelow, J. S.; Wieckowski, A. *Phys. Chem. Chem. Phys.* **2007**, *9*, 2654.
(22) Eckardt, M. J.; File, S. E.; Gessa, G. L.; Grant, K. A.; Guerri, C.; Hoffman, P. L.; Kalant, H.; Koob, G. F.; Li, T.-K.; Tabakoff, B. *Alcohol.: Clin. Exp. Res.* **1998**, *22*, 998.
(23) Kamitaka, Y.; Tsjimura, S.; Setoyama, N.; Kjino, T.; Kano, K. *Phys. Chem. Chem. Phys.* **2007**, *9*, 1793.
(24) Hagen, J.; Socaciu, L. D.; Roux, J. L.; Popolan, D.; Bernhardt, T. M.; Woste, L.; Mitric, R.; Nolak, H.; Bonacic-Koutecki, V. *J. Am. Chem. Soc.* **2004**, *126*, 3442.
(25) Chatterjee, M.; Chatterjee, A.; Ghosh, S.; Basumallick, I. *Electrochim. Acta* **2009**, *54*, 7299.

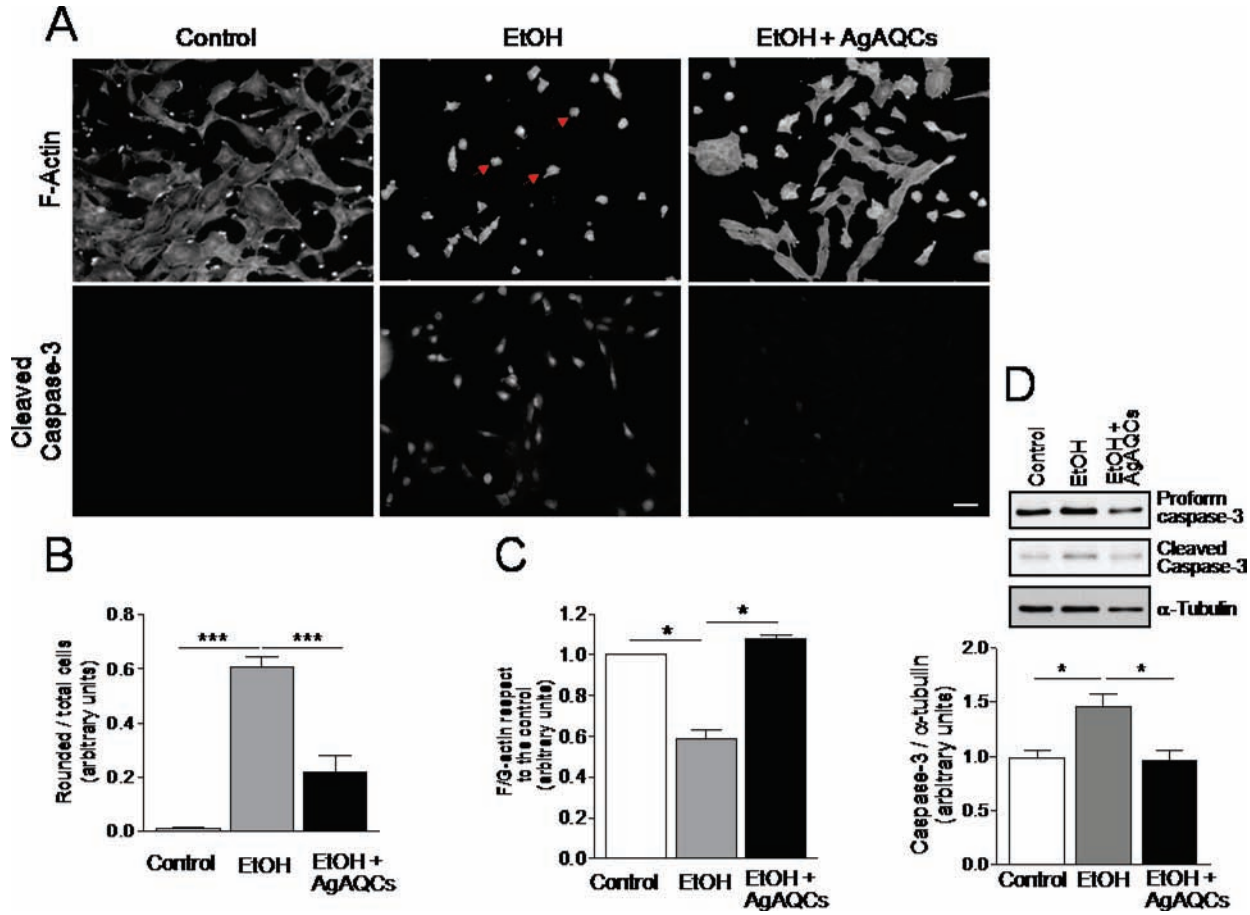

Figure 3. AgAQCs prevent ethanol-induced alterations in the cellular morphology and the actin cytoskeleton organization. (A) Ethanol-exposed cells (200 mM) were incubated in the absence or in the presence of AgAQCs (1 μg/mL) for 30 min. The cell shape and the actin cytoskeleton organization (F-actin) were evaluated under the epifluorescence microscope using FITC-phalloidin. Activation of apoptosis was also evaluated using immunofluorescence by the presence of cleaved caspase-3 in the cell nucleus. Scale bar, 20 μm. (B) Quantitative analysis of results shown in panel A. Statistical significance according to one-way ANOVA, (***) $p \leq 0.001$. (C) Globular (G) and filamentous (F) actin pools from unexposed (control) or ethanol-exposed cells in the absence (EtOH) or in the presence of AgAQCs (EtOH + AgAQCs), were analyzed by immunoblotting using anti-actin antibodies (see a representative experiment in Figure S4, Supporting Information). Statistical significance according to the Student's *t* test, (*) $p \leq 0.05$. (D) Immunoblot analysis of unexposed (control) and ethanol-exposed cells incubated in the absence (EtOH) or in the presence of AgAQCs (EtOH + AgAQCs). Caspase-3 activation was detected using antibodies against caspase-3 fragments (19 and 17 kDa; see also Figure S5B, Supporting Information). α-Tubulin was used as a loading control. Bars represent the ratio between positive apoptotic cells with respect to total cells. Statistical significance according to one-way ANOVA, (*) $p \leq 0.05$.

reported by Minteer's group,[26] but 3 orders of magnitude lower than that reported by Gouveia-Caridade et al.[27]

3.2. AgAQCs Prevent Ethanol-Induced Alterations in Cultured Mammalian Cells. Since AgAQCs electrocatalize *in vitro* the oxidation of ethanol at very low potentials and in a physiological medium, we explored whether AgAQCs could prevent or mitigate some well-known cellular alterations that are observed in cells exposed to ethanol. Our previous studies in primary cultures of rat astrocytes showed that long-term ethanol exposure (chronic treatment) perturbs the organization of the actin cytoskeleton, which is visualized by a decrease of actin stress fibers and the formation of peripheral actin rings.[15,16] However, in this present work we had to establish another cellular model to evaluate first the cytotoxicity and then the subcellular alterations induced by ethanol at shorter periods of time (acute treatment). In a variety of clonal cell lines, we evaluated three parameters which are representative of the acute ethanol toxicity: (1) the appearance of a rounded cell shape as a consequence of the abnormal rearrangement of the actin cytoskeleton, (2) alterations in the ratio between filamentous (F-actin) and globular (G-actin) actin, and (3) the induction of apoptosis. We finally chose NRK cells because of their higher sensitivity to ethanol exposure (200 mM, 30 min; Figures S3−S5, Supporting Information), as well as tolerance to AgAQCs concentrations up to 1 μg/mL (~0.3 mM) (Figures S6 and S7, Supporting Information). Next, we tested the potential cytoprotective effect of AgAQCs. Thus, NRK cells were exposed to ethanol (200 mM) in the presence or absence of AgAQCs (1 μg/mL) (Figure 3 and Figure S8, Supporting Information).

Ethanol significantly increased the number of cells that presented a rounded morphology (red arrows) and a disorganized actin cytoskeleton (Figures 3A,B and S4). In contrast, in the presence of AgAQCs, the number of ethanol-induced rounded cells decreased (Figures 3A,B and S8), and most exhibited a normal actin organization (Figure 3A). We also measured the ratio between F-actin and G-actin to evaluate the integrity of the actin cytoskeleton.[28] Ethanol significantly reduced the F-/G-actin ratio (Figures 3C and S4C,D), but importantly the total amount of actin remained unaltered (Figure S4D, INPUT). As

(26) Topcagic, S.; Minteer, S. D. *Electrochim. Acta* **2006**, *51*, 2168.
(27) Gouveia-Caridade, C.; Pauliukaite, R.; Brett, C. M. A. *Electrochim. Acta* **2008**, *53*, 6732.

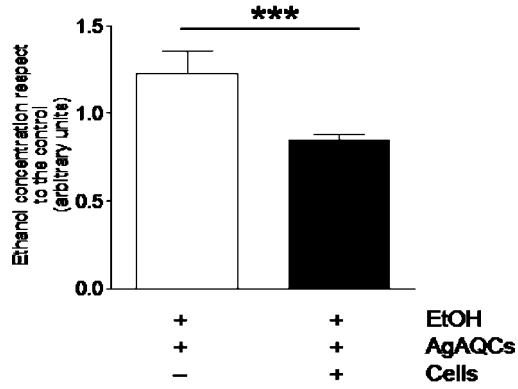

Figure 4. AgAQCs decrease the concentration of ethanol in the culture medium only when cells are present. Determination of the ethanol concentration in the cell culture medium that contained ethanol (200 mM) and 1 μg/mL AgAQCs with (+) or without (−) NRK cells. Statistical significance according to the Student's *t* test, (***) $p \leq 0.001$.

expected, AgAQCs prevented the ethanol-induced reduction of the F-/G-actin ratio (Figure 3C).

Since ethanol induces oxidative stress and cell death by apoptosis,[29−31] we then explored whether AgAQCs could also prevent it. The activation of caspase-3 is a key mediator and a well-established cellular marker of apoptosis in mammalian cells.[30,32] The induction of apoptosis was examined by immunofluorescence and immunoblotting based on the formation and nuclear localization of proteolytic fragments of caspase-3. Cleaved caspase-3 appeared in the nucleus 30 min after the ethanol exposure (Figures 3A and S5A). Caspase-3 fragments were also resolved by immunobloting (Figures 3D and S5B,C). When ethanol-exposed cells were incubated with AgAQCs, both the proteolysis and the nuclear localization of caspase-3 were prevented (Figure 3A,D).

Therefore, our data showed that AgAQCs prevent the cellular alterations produced by ethanol. Strikingly, this protection was obtained only when ethanol and AgAQCs were both added simultaneously to cells. When one was added before the other, the cytoprotection was not produced (data not shown). This result indicates that cytoprotection by AgAQCs against ethanol results from the combined action of sub-nanoclusters and cells (see below).

3.3. AgAQCs Oxidize Ethanol Only in the Presence of Cells. Regarding the catalytic properties of AgAQCs for the electro-oxidation of ethanol, we examined whether the cytoprotection mediated by AgAQCs in NRK cells takes place by decreasing the ethanol present in the culture medium. To this aim, we measured the final ethanol concentration in the culture medium of cells exposed to ethanol (200 mM) in the absence and in the presence of AgAQCs (1 μg/mL). As shown in Figure 4, ethanol significantly diminished in the culture medium when AgAQCs were present.

Interestingly, this decrease was produced only when the culture medium was in contact with cells, which is in accordance with the observation that the prevention mediated by AgAQCs against the reported ethanol-induced alterations occurred only when ethanol and AgAQCs were both added to cells simultaneously. Therefore, our data suggest that AgAQCs need the presence of cells to be cytoprotective.

3.4. AgAQCs Are Also Cytoprotective in Rat Astrocytes. The protection by AgAQCs against ethanol toxicity in cells expands its conceivable application in the ethiopathogenesis of alcohol abuse. Ethanol is a teratogen that affects the development of the nervous system, where both neurons and glial cells are perturbed.[13,31] Among other alterations, ethanol disorganizes the actin cytoskeleton in primary cultures of rat astrocytes.[15,16] Therefore, we examined whether AgAQCs could protect newborn rat astrocytes from the effects of acute ethanol exposure. Thus, changes in the cell shape and in the actin cytoskeleton organization were examined. However, to produce these alterations in rat astrocytes, it is necessary to use a higher ethanol concentration than for NRK cells (500 vs 200 mM, respectively). Thus, primary cultures of rat astrocytes were incubated with AgAQCs at 0.5 (data not shown) or 1 μg/mL for 30 min (Figure 5). At both AgAQCs concentrations, cells showed no alteration either in the cell shape or in the actin cytoskeleton organization. As expected, ethanol induced alterations in the cell shape and in the actin cytoskeleton organization, but alterations of both were significantly prevented when astrocytes were co-incubated with AgAQCs (Figure 5). These results indicate that, as in NRK cells, AgAQCs are also cytoprotective for astrocytes exposed to ethanol.

3.5. AgAQCs Prevent Alterations Induced by Other Alcohols but Not Those Caused by H_2O_2. We next explored whether AgAQCs also protect cells from harmful effects triggered by other alcohols such as methanol or butanol as well as the catalytic properties of AgAQCs for the electro-oxidation of these other alcohols (Figure 6).

NRK cells were exposed to methanol (50 mM) or butanol (25 mM) for 30 min, and the cell shape and the organization of the actin cytoskeleton were analyzed. Both alcohols induced similar actin cytoskeleton rearrangements like those produced by ethanol with the concomitant change in the cell morphology (Figure 6A). These results are in accordance with previous data obtained in other cell lines.[33] The number of methanol- or butanol-exposed cells with a rounded morphology and altered actin cytoskeleton significantly decreased when cells were co-incubated with the respective alcohol and AgAQCs (Figure 6A and B). Therefore, the protective effect of AgAQCs is not restricted to ethanol, but it also occurs for other primary alcohols.

We next examined whether AgAQCs are cytoprotective against toxic agents other than alcohols. To this aim, we used hydrogen peroxide (H_2O_2), which like ethanol also induces oxidative stress.[34] NRK cells exposed to H_2O_2 (250 μM for 30 min) produced severe changes in both the actin cytoskeleton organization and the cell shape, neither of which was prevented by AgAQCs (Figure 7A,B).

Interestingly, electro-oxidation of methanol and butanol also occurred in acidic and basic media (data not show) as well as in PBS at the same low potentials, $\lesssim +200$ mV registered for ethanol (compare Figures 2C and 6C,D). In contrast, electroreduction of H_2O_2 in acidic medium or in PBS occurred at much more negative potentials (Figure 7C,D). In acidic medium (Figure 7C), electro-reduction occurred from -0.6 V, a similar value to that obtained in the curve of H_2O_2 in N_2 after the O_2

(28) Lázaro-Diéguez, F.; Aguado, C.; Mato, E.; Sánchez-Ruíz, Y.; Esteban, I.; Alberch, J.; Knecht, E.; Egea, G. *J. Cell Sci.* **2008**, *121*, 1415.
(29) Zhou, Z.; Sun, X.; Kang, Y. J. *Am. J. Pathol.* **2001**, *159*, 329.
(30) Ieraci, A.; Herrera, D. G. *PLoS Med.* **2006**, *3*, 0547.
(31) Harper, C. *Hum. Exp. Toxicol.* **2007**, *26*, 251.
(32) Köhler, C.; Orrenius, S.; Zhivotovsky, B. *J. Immunol. Methods* **2002**, *265*, 97.
(33) Komati, H.; Naro, F.; Mebarek, S.; De Arcangelis, V.; Adamo, S.; Lagarde, M.; Prigent, A. F.; Némoz, G. *Mol. Biol. Cell* **2005**, *16*, 1232.
(34) Han, D.; Hanawa, N.; Saberi, B.; Kaplowitz, N. *Free Rad. Biol. Med.* **2006**, *41*, 627.

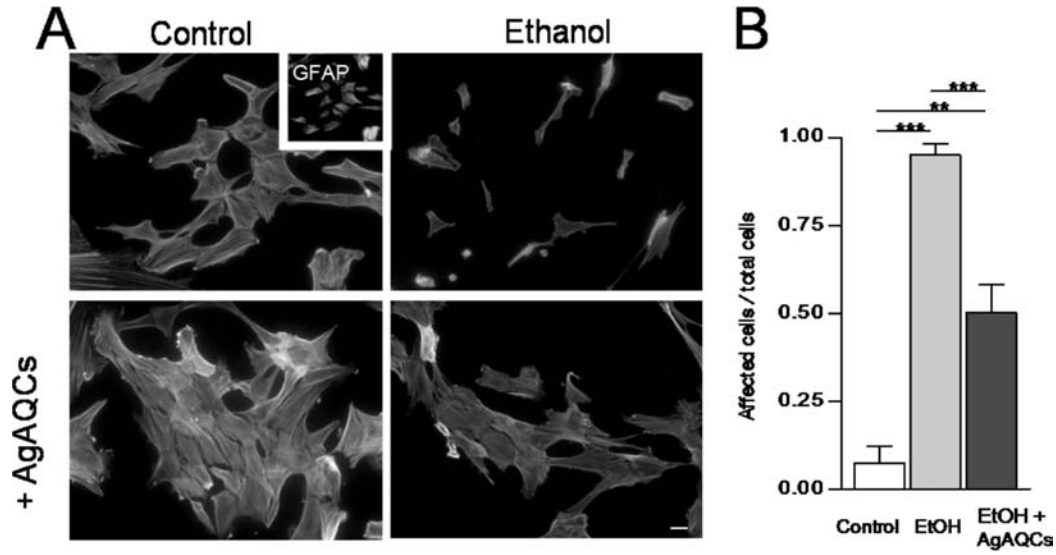

Figure 5. AgAQCs prevent the deleterious effects induced by ethanol in primary cultures of rat astrocytes. (A) Unexposed (control) and ethanol (500 mM)-exposed astrocytes were incubated with AgAQCs (1 μg/mL) for 30 min and stained with FITC-phalloidin. Immunostaining with glial fibrillar acidic protein (GFAP) antibodies identified astrocytes (inset in panel A). Scale bar, 20 μm. (B) Quantitative analysis of the results shown in panel A, in which we determined the ratio of ethanol-affected cells with respect to total cells (immunostained for GFAP). Statistical significance according to the Student's *t* test, (**) $p \leq 0.01$ and (***) $p \leq 0.001$.

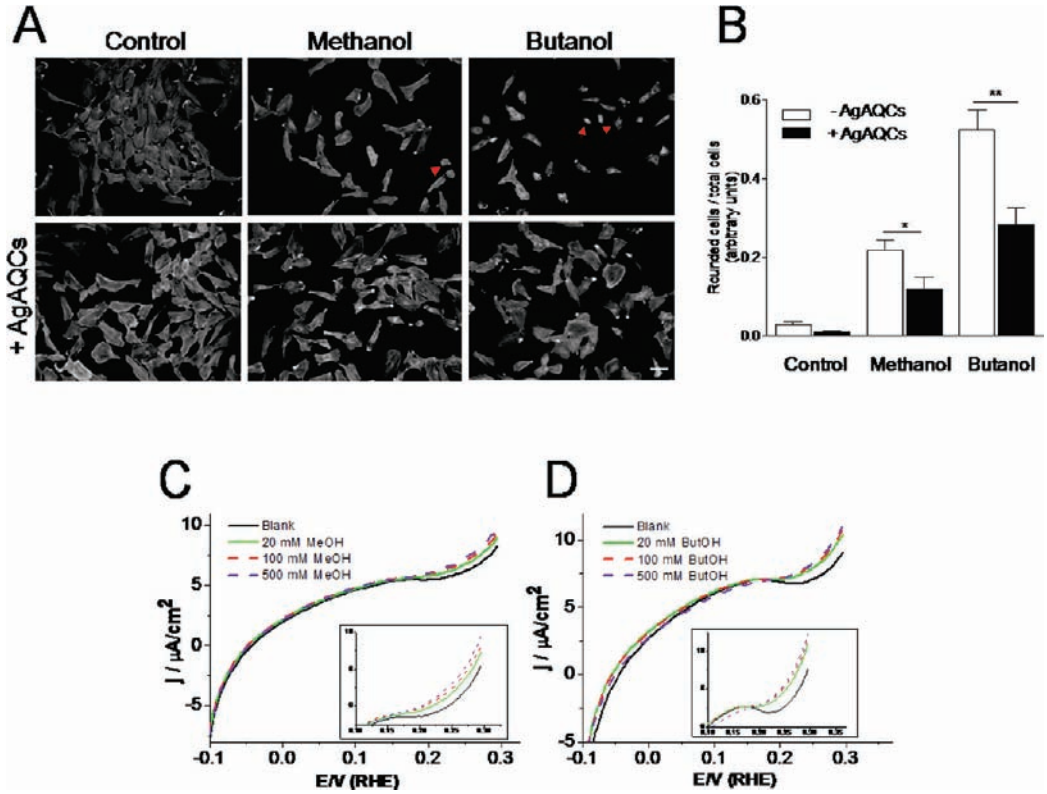

Figure 6. AgAQCs also protect cells against methanol or butanol exposure. (A) Unexposed cells (control) and cells exposed to methanol (50 mM) or butanol (25 mM) were incubated in the absence or in the presence of AgAQCs (1 μg/mL) for 30 min. Actin cytoskeleton was visualized using FITC-phalloidin. Scale bar, 20 μm. (B) Quantitative analysis of the results shown in panel A. The ratio of altered cells (red arrowheads in panel A) with respect to total cells is indicated. Statistical significance according to one-way ANOVA, (*) $p \leq 0.05$ and (**) $p \leq 0.01$. (C,D) Voltammograms of the oxidation at AgAQCs/GC electrode at a sweep rate of 50 mV s^{-1} of 20, 100, and 500 mM methanol and butanol in PBS.

elimination. In contrast, electro-reduction of H_2O_2 in PBS (Figure 7D) showed two well-defined steps when both H_2O_2 and O_2 were present. The first sharp electrocatalytic current peak (peak 1) corresponds to O_2 and the second one (peak 2) to H_2O_2 reduction. The latter in PBS was much less favorable than in acidic medium, where the current decreased 2 orders of magnitude and the reduction peak was shifted to more negative potentials (~ -0.9 V in PBS vs -0.6 V in acidic medium).

3.6. Potential Mechanisms for Ethanol Electrocatalysis by AgAQCs in Cultured Mammalian Cells. Our results show that deleterious effects of ethanol in culture cells can be prevented by AgAQCs, which promote a decrease in the ethanol concen-

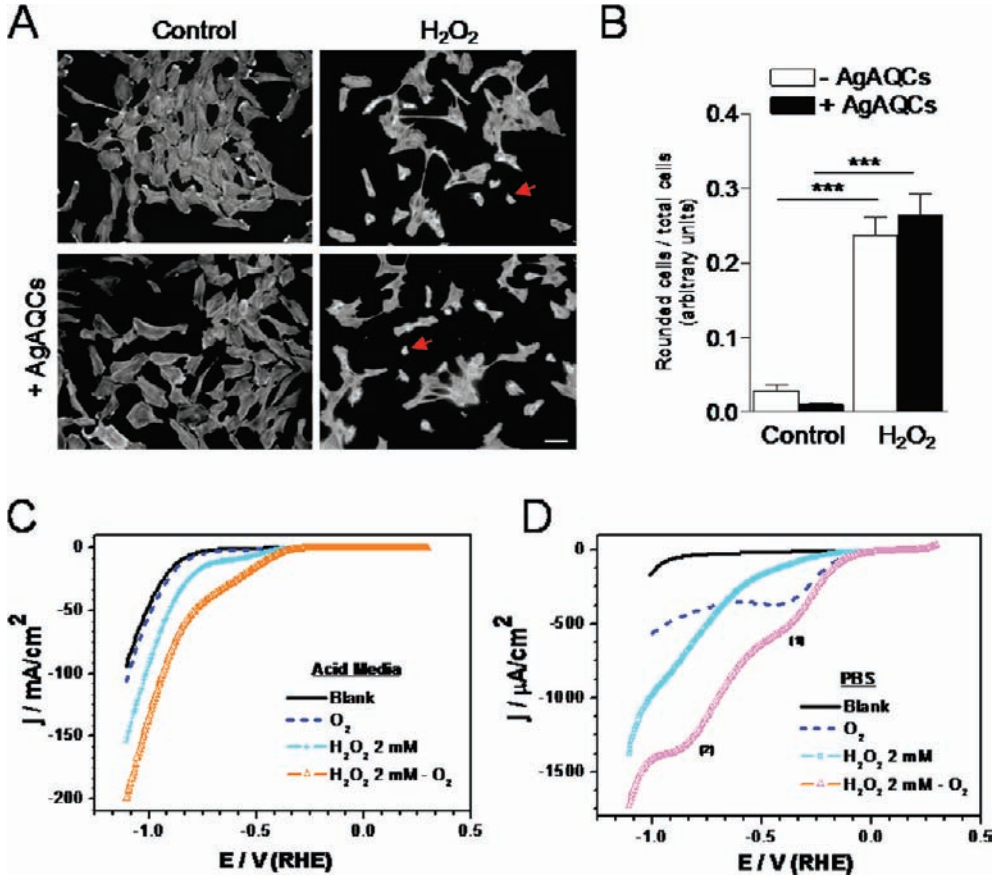

Figure 7. AgAQCs do not protect cells against H_2O_2. (A) Unexposed (control) and H_2O_2 (250 μM)-exposed NRK cells were incubated with AgAQCs (1 μg/mL) for 30 min. The actin cytoskeleton was visualized using FITC-phalloidin. Red arrows show some representative rounded cells caused by H_2O_2. Scale bar, 20 μm. (B) Quantitative analysis of the results shown in panel A. Statistical significance according to one-way ANOVA, (***) $p \leq 0.001$. (C,D) Forward reductive sweeps of cyclic voltammograms showing the H_2O_2 electrocatalysis in a AgAQC/GC electrode at a sweep rate of 50 mV s^{-1} with or without H_2O_2 in either acidic medium (C) or PBS (D) in N_2 atmosphere without H_2O_2 (black line), in N_2 atmosphere with 2 mM H_2O_2 (turquoise line), or in O_2 atmosphere with 2 mM H_2O_2 (orange line). For identification of peaks (1 and 2) in PBS, another sweep in O_2 atmosphere without H_2O_2 (blue dotted line) was added in panel D.

tration present in the culture medium. Since this decrease takes place only when ethanol, AgAQCs, and cells are all present in the assay, it is reasonable to hypothesize that cell membranes have to be the suppliers of the electrical potential required by AgAQCs to mediate their prevention. The observation that, under physiological conditions, AgAQCs electrocatalyze ethanol oxidation at a potential and current densities of the same order of magnitude of those measured in the plasma membrane in excitable and non-excitable cells seems to support such a hypothesis.[35] Moreover, notice that both parameters (membrane potentials and current densities) are of the same order of magnitude as those reported for electrochemical events measured in other organelles, such as mitochondria.[36]

AgAQCs are planar structures[18] that tend to attach to interfaces of model membranes like surfactant films in microemulsions.[10,11] Therefore, it is reasonable to hypothesize that the association of Ag clusters with the plasma membrane could provide suitable conditions for the electrocatalysis of ethanol oxidation. We were unable to provide any direct evidence for the uptake and subcellular localization of AgAQCs in cells because they are too small to be resolved by conventional electron microscopy for biological samples. In addition, although AgAQCs emit fluorescence,[10] their low emission wavelengths and low concentration used in our study did not allow us to detect them either in cells or in the culture medium.

As can be seen, the minimum potential for ethanol oxidation in PBS (minimum free energy to drive the reaction) is +200 mV. Therefore, by comparison of the same electrocatalytic reaction in the electrochemical and NRK cells, it is possible to estimate the minimum potential (minimum free energy) to drive the reaction at the cell membrane side (outer/inner) where electrocatalysis could take place. From the observation that electro-oxidation of alcohols occurs in NRK cells, the value of the oxidative outer plasma hemimembrane potential in these cells should then be $\varphi_{out} \geq +200$ mV vs RHE. This value is larger than the effective redox potential of the oxidizing extracellular environment ($\sim$0 V vs RHE).[37] However, it has to be taken into account that the concept of effective redox potentials in cells is not well defined and represents only an average value that varies over a broad range and largely depends on the cell type, oxidative stress conditions, etc.[38] On the other hand, surface and dipole potentials cause the electrical membrane potential to deviate from the mean values of the intra-

(35) Kandel, E. R.; Schwartz, J. H.; Jessell, T. M. *Principles of Neural Science*, 4th ed.; McGraw-Hill: New York, 2000; pp 126−127.
(36) Bockris, J. O. M.; Reddy, A. K. N. *Modern Electrochemistry 2B*; Kluwer Academic: New York, 2000; p 1967.
(37) Moriarty-Craige, S. E.; Jones, D. P. *Annu. Rev. Nutr.* **2004**, *24*, 481.
(38) Grigory, G.; Martinovich, G. G.; Cherenkevich, S. N.; Sauer, H. *Eur. Biophys. J.* **2005**, *34*, 937.

and extracellular compartments.[39] From the value of the oxidative outer hemimembrane electrical potential estimated above, and taking into account the usual values for transmembrane potentials (φ_{trans}) measured in nonexcitable mammalian cells (from −30 to −90 mV),[35] one could then estimate the value of the reductive inner plasma hemimembrane potential, $\varphi_{in} = \varphi_{out} - \varphi_{trans} > +200 - 30$ (or − 90) mV = +170 (or +110) mV. According to this estimation, the minimum potential required at physiological conditions for the reduction of H_2O_2 (−900 mV) is far from that provided by the less positive inner hemimembrane potential value ($\varphi_{in} = +110$ mV). Therefore, AgAQCs do not electrocatalyze the reduction of H_2O_2 in cells only because they cannot provide the required potential for leading the electrocatalytic reaction.

4. Conclusions

Herein we show that injuries produced by ethanol in cultured mammalian cells are prevented by AgAQCs. AgAQCs electrocatalyze the oxidation of ethanol under physiological conditions at very low potentials. The protection in mammalian cells provided by Ag clusters is observed only when ethanol, AgAQCs, and the cells are all present in the assay. We postulate that cells provide the electrical potential required by AgAQCs to mediate their cytoprotective role against ethanol, which suggests that cells could act as true power sources for the electrocatalysis of biochemical reactions. Finally, the protection provided by AgAQCs against ethanol in a cellular model of FAS (rat astrocytes exposed to ethanol) widens their potential use in the prevention of the effects of ethanol on the central nervous system.

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Supporting Information Available: Experimental methods and supplementary figures.This material is available free of charge via the Internet at http://pubs.acs.org.

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(39) Clarke, R. J. *Adv. Colloid Interface Sci.* **2001**, *89−90*, 263.