

Effects of Pluronic F68 Micellization on the Viability of Neuronal Cells in Culture

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ABSTRACT: Triblock copolymers with surface-active properties, referred to as Pluronic, have shown potential medical applications such as drug delivery to selective targets in the human body. In particular, the transport of anti-inflammatory substances to the brain is required for illness treatment, thus the study of delivery agents that cross the blood–brain barrier is relevant. In this article we study the effects of the micelle formation on the morphologic and cytotoxic properties of Pluronic F68. We determinate the critical micellar concentration (CMC) by standard tensiometric and absorbance measurements, and also we analyze the morphology of polymers by atomic force microscopy. Our observations indicate that the morphological properties of F68 are drastically modified in the CMC range, as well as the ability to increase the viability of neuroblastoma cells maintained under culture conditions, as compared with nontreated cells. Our conclusions highlight the close correlation between morphological and physicochemical properties of Pluronic, which must be further understood in order to achieve highly controlled pharmacological uses. © 2013 Wiley Periodicals, Inc. *J. Appl. Polym. Sci.* 130: 2159–2164, 2013

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INTRODUCTION

The block copolymers consist of hydrophilic poly(ethylene oxide) (PEO) and hydrophobic poly(propylene oxide) (PPO) blocks arranged in tri-block structure: PEO–PPO–PEO. These commercially available noncytotoxic nonionic agents are commonly referred to as Pluronic or poloxamers are a class of surface-active, amphiphilic molecules.¹ These have an extensive range of applications in the medical field such as drug solubilization, controlled drug release.^{2–4} Moreover, Pluronic have recently attracted a great deal of attention as excellent membrane sealants with low toxicity.^{5–7} Of particular interest is poloxamer 188 (Pluronic F68), an approximately 8400 g/mol poloxamer of the form PEO₇₆-PPO₂₉-PEO₇₆ that has been proven as a surfactant sealing agent for permeabilized lipid bilayers.⁵ When membrane permeability increases dramatically in the case of trauma or infectious diseases, the natural pathway for maintaining the normal permeability of cell membranes can be overwhelmed, rendering it incapable of arresting leakage in these permeabilized membranes. The efficacy of F68 as a cell membrane sealant was first shown when the polymer reduced leakage of carboxyfluorescein dye from loaded cells after

electroporation.⁵ Further more F68, has been proven to be a successful sealing agent for various permeabilized cells, including long protection of both neuronal and non-neuronal cells from cell death by resealing the injured cell membrane and enhancing the functional recovery of injured cells by either electric shock, thermal shock, excitotoxic, or oxidative agents.⁸

Recently, it was demonstrated that F68 provided an acute recovery of axonal function in spinal cord injury models.^{9,10} In fact, the treatment both restores membrane integrity acutely and restores viability of mechanically injured neuronal cells to control levels at 24 hours postinjury,¹¹ and both *in vitro* and *in vivo* studies have demonstrated the effectiveness of F68 in the recovery of damaged cell membranes.⁹ Although these studies indicate the effectiveness of poloxamers as a pharmaceutical agent, little is known about the physicochemical mechanism that mediates molecular interactions between polymers and cells.¹²

Morphological changes and physicochemical parameters suggest to serving as an effective model for the functions of the outer protective agent F68. In particular, we suggest that the

protective effect is mainly because of biological interactions and physicochemical mechanism between the potential protective agents and cells. These are likely to occur by absorption of the reagents onto the cell membrane with the subsequent formation of a layer, which protects cells from damage and loss of nutrients. Thus, Pluronic F68 would reduce the surface tension of the culture medium, favoring cell–macromolecules contact, with a consequent increase in the viability of neuroblastoma.

In this article, we study the formation of F68 supramolecular aggregates and its influence in the cell viability of human neuroblastoma. For this purpose, we first measured the critical micellar concentration (CMC) and morphology of F68 in aqueous solution as a function of the molar concentration. Second, we evaluated the cell viability in an experimental colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay,¹³ also as a function of the F68 molar concentration. We observed that above and below the CMC, the morphology, surface tension, and cell viability present significant changes that are coherent with the modification of the encapsulation performance into the plasmatic membrane.¹⁴

EXPERIMENTAL

Determination of CMC

The values of the CMC of Pluronic F68 (Sigma Aldrich) were determined using different solvents and Sudan III. The dry Sudan (Kahlbaum, Germany) was added to Pluronic solutions at different surfactant concentrations. When using nanopure water, the mixtures were shaken during 5 min at 37°C and then centrifuged at 7000 rpm for the separation of the aqueous phase from solid Sudan particles. The condition of a solubilized Sudan in the micellar phase was determined by spectrophotometry at 507 nm using spectrophotometer detector. A similar experiment was carried out using human blood serum (48 hours at 37°C), collected in potassium/EDTA and potassium citrate/EDTA tubes. For tensiometric analysis, a tensiometer from Krüss was used, and the measurements were carried out using variable F68 concentrations in solutions of 20 mL of ultrapure water at 37°C, and agitated for 5 hours.

Morphological Analysis by Atomic Force Microscopy

Atomic force microscopy (AFM) is an excellent, nondestructive, and high-resolution technique for investigating the surface structures of adsorbed films.^{15,16} F68 solutions were prepared in the $[0.4\text{--}10.0] \times 10^{-4} M$ concentration range and deposited on 0.4-mm-thick crystalline Si(100) wafer (Virginia Semiconductor), coated by its native oxide layer. Prior to F68 deposition, we cleaned the silicon substrate by immersing it into a solution of sulfuric acid and hydrogen peroxide (70% H_2SO_4 + 30% H_2O_2) at 90°C for 30 min, providing an hydrophilic substrate surface.¹⁸ Previous analysis of X-ray reflectivity curves,¹⁷ indicated that the thickness of the SiO_2 coating was 12 Å. This cleaning procedure does not remove the native oxide layer and provides very reproducible substrates. After cleaning, the substrates were stored in ultrapure water (Merk) and then dried in a jet of dry nitrogen before adsorbing the F68 film. Pluronic F68 was deposited on the silicon substrate in an amount of 30 μL for each of those solutions and substrates. After drying at

room temperature, the topography of the samples was studied by AFM in the contact mode, at room temperature ($\sim 25^\circ C$).

Determination of Cell Viability

The neuroprotective effect of F68 on viability of cultured neural models (SH-SY5Y; 37°C) was assessed by the MTT method. Increasing concentrations of Pluronic F68 were applied to cells in the culture medium pregrown for two weeks in MEM/F12/10% FCS medium, and differentiated with retinoic acid (6000 cells per well in culture plates of 12×8 cm). After 24 hours, the cells were analyzed for viability by MTT assay.¹³ MTT reagents from Sigma and the procedure were carried out as described in previous work.¹⁹ Both Pluronic and culture media were sterilized by filtration through filters of 0.2 μm . An appropriate amount of antibiotics were added to culture medium.

For MTT, cells were incubated with a solution of a total volume of 100 μL , which contains 5 mg/mL of MTT in PBS and added 10% of the total volume at 37°C for 4 hours. Then, a solution buffer pH 7 of 200 μL total [50% Dimethylformamide Sigma; 20% SDS] was added. After overnight incubation with extraction buffer at 37°C, cell solutions were used to measure absorbance at test wavelength of 550 nm and a reference wavelength (background) of 650 nm.

RESULTS AND DISCUSSION

Determination of CMC

In general, the values of CMC of Pluronic in aqueous solution differ considerably, depending on the procedure used for their determination.^{20–22} We estimated the CMC value at 37°C, on one side, by studying the absorbance of Sudan III in F68 in three different liquid matrices and, on the other side, by tensiometric analysis. For both measurements, the CMC range was determined by the inflection in the absorbance and tension curves, as it represents the formation of micelles.^{14,21}

As observed in Figure 1, the measured CMC ranges for F68 in nanopure water determined by the spectrophotometric and tensiometric analysis were $[0.9\text{--}2.3] \times 10^{-4} M$ [Figure 1(a), lower curve] and $[0.7\text{--}3.5] \times 10^{-4} M$ [Figure 1(b)], respectively. Although these results also agree with similar experiments,⁵ discrepancies with reported values for F68 arise when using different methods or different chemical environments, i.e., when measured using pyrene at 37°C, the CMC give values within $[4.8\text{--}5.9] \times 10^{-4} M$.²⁴ In order to obtain an estimated value of CMC for F68 under biological conditions, we used human plasma instead of nanopure water. As shown in Figure 1(a), the estimated CMC ranges for human plasma in Potassium citrate/EDTA (5 mL/10, mg) and Potassium/EDTA (5.0 mL/10.8 mg), resulted $[0.8\text{--}2.6] \times 10^{-4} M$ and $[0.9\text{--}4.9] \times 10^{-4} M$, respectively. These values slightly differ from the range observed using pure water suggesting that the hydrophilic corona of the F68 is not strongly affected by the adhesion of blood serum proteins, and thus micelles remain stable in the human blood plasma.^{25,26}

Morphological Analysis of Polymeric Films

AFM was used to investigate the morphological changes of F68 aggregates in water induced by different concentrations of F68.

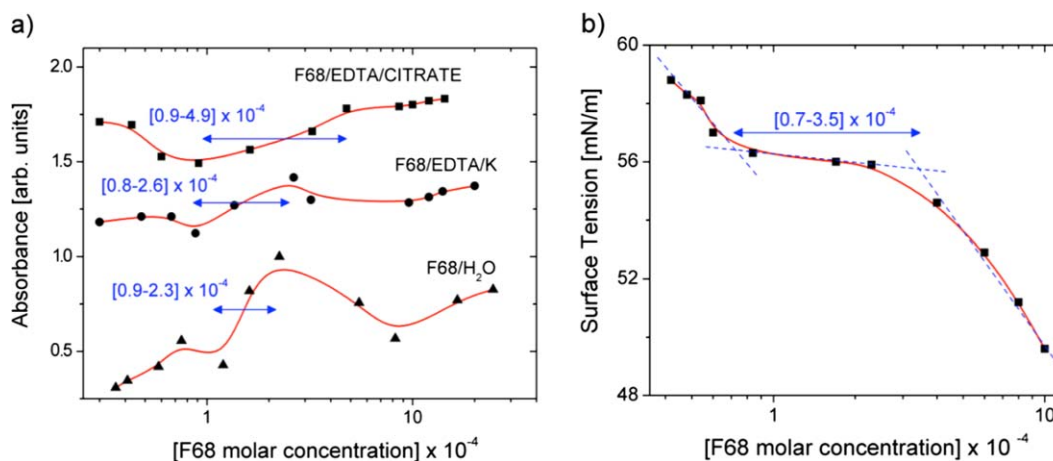


Figure 1. (a) Absorbance of Sudan III at 507 nm using spectrophotometer detector, in function of F68 concentration. The solvents solutions used include human plasma in Potassium citrate /EDTA (5 mL/10.0 mg), human plasma in Potassium/EDTA (5.0 mL/10.8 mg) and pure water. (b) Surface tension of pure water in function of F68 concentration. The CMC range was determined by the first inflection observed in the response curve. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

The samples were disposed as films on hydrophilic silicon substrates. The observed structures and morphologies were stable at room temperature and no morphological changes were observed after multiple AFM scans in different regions within each sample. The morphology of Pluronic F68 showed in Figure 2 was measured 24 hours after filtration. First, the morphology was characterized by sphere aggregates (not showed) and then slowly evolved to dendritic structures.

A morphological transition is observed from small aggregates or clusters for low concentrations, to a final supramolecular dendritic structure for high concentrations. This also induced a change in the area covered by the F68 film in the AFM images. The stripes present in some AFM images are because of the scrape of the in-contact tip on the polymer. Figure 3 shows the percentage of the F68 coverage together with the surface tension presented in Figure 1(b), as a function of the molar concentration.

It is worth to note that the F68 coverage curve obtained from AFM measurements shows a maximum and a minimum in the CMC region ($[0.7-3.5] \times 10^{-4} M$). This is probably because of the structural transitions associated with the rearrangement of the polymeric molecules. The minimum observed in the coverage at around $2.0 \times 10^{-4} M$ might correspond to the formation of micellar aggregates, which reduces the contact area between the hydrophilic silicon substrate and the molecular aggregates. This effect also produces a relatively constant value of the surface tension, though the concentration of F68 increases.¹⁴

Viability of Human Neuroblastoma Cells

In order to test whether F68 induces neuroprotection in a concentration-dependent fashion, we analyzed the cell viability of the cultured human neuroblastoma cells previously differentiated with retinoic acid for two weeks at 37°C. For this purpose, F68 aliquots at three different concentration ranges were applied to predifferentiated SH-SY5Y cells: (i) low concentrations $[0.42-10.0] \times 10^{-4} M$, (ii) medium concentrations $[12.0-24.0] \times$

$10^{-4} M$ and (iii) high concentrations $[72.0-120.0] \times 10^{-4} M$. The cells were incubated with F68 for 24 h, and the cell viability was analyzed using the MTT method.

As shown in Figure 4(a), at low F68 concentrations, we observed a maximal increase of 68% in cell viability with respect to the control case (absence of micelles). At higher concentrations, we observed a continuous decrease of the viable cells down to 10%, revealing the cytotoxicity of F68 at high concentration.²⁷ Enormous variability in cell responses to F68 exists across different cell types,²⁸ and indeed some mammalian cell lines have exhibited slight toxicity.²⁹ In the case of human neuroblastoma SH-SY5Y cells our estimated ratio of the specific toxic effects and the therapeutic effect (therapeutic index) was 134, which suggests that F68 may be considered safe.³⁰

Effects of CMC on Cell Viability

With the aim of study the effect of the micelles formation on the cell viability, we compare the tension energy and the viability behaviors. The surface tension (γ) is defined as the partial derivate of the Gibbs free energy (G) as a function of the area (A), at a constant number of particles (n), temperature (T), and pressure (P) [eq. (1)]. Thus G can be estimated from the data of γ and the AFM coverage area (A), using eq. (2). The numerical derivate and integration were performed by usual finite calculation methods using the F68 concentration (C) as the independent variable.

$$\gamma = \left(\frac{\partial G}{\partial A} \right)_{n,T,P} \quad (1)$$

$$G = \int \left(\frac{dA}{dC} \right) \gamma dC \quad (2)$$

Figure 4(b) shows the correlation between the cell viability and the module of the tension energy (G) as a function of the F68 concentration. It is worth to note that a fast increase and a

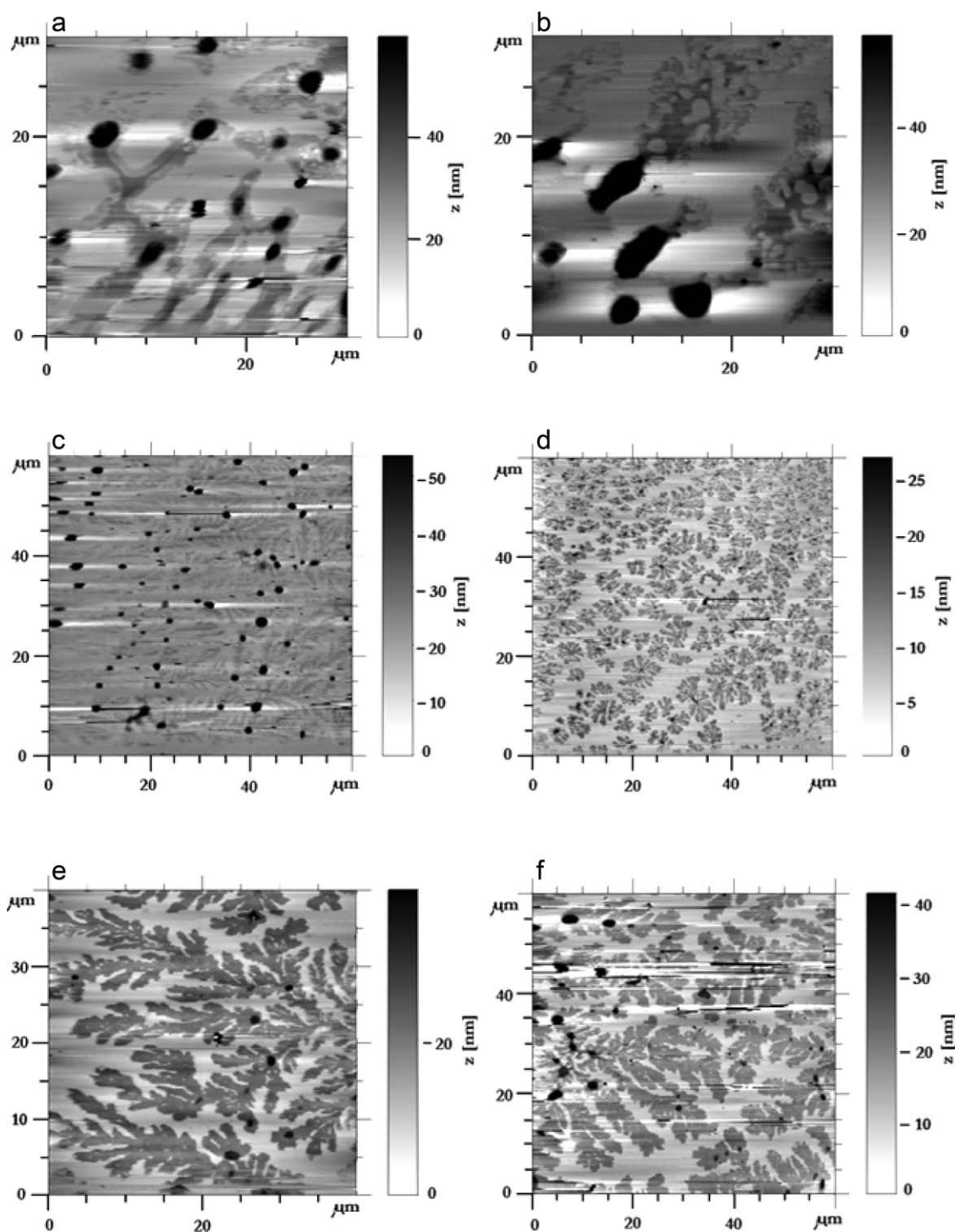


Figure 2. Set of six AFM images obtained in contact mode on the F68 films formed on the hydrophilic Si/SiO₂ substrate at room temperature ($\sim 25^{\circ}\text{C}$). The images are labeled for each of the molar concentration of F68 are as follows: (a) $0.50 \times 10^{-4} \text{M}$; (b) $0.62 \times 10^{-4} \text{M}$; (c) $2.00 \times 10^{-4} \text{M}$; (d) $4.00 \times 10^{-4} \text{M}$; (e) $6.00 \times 10^{-4} \text{M}$; and (f) $10.00 \times 10^{-4} \text{M}$.

maximum in both curves at a value of around $1.2 \times 10^{-4} \text{M}$ are observed. Around $2.0 \times 10^{-4} \text{M}$, the two curves split with a decrease in cell viability and a slow increase in the tension energy because of higher suspension density. This evidence suggests that the increase in cell viability is directly induced by a

minimization of the surface energy of the F68/water system, because of the micelles formation.

Figure 5 shows the cell viability as a function of the surface tension, extracted from the data shown in Figures 3 and

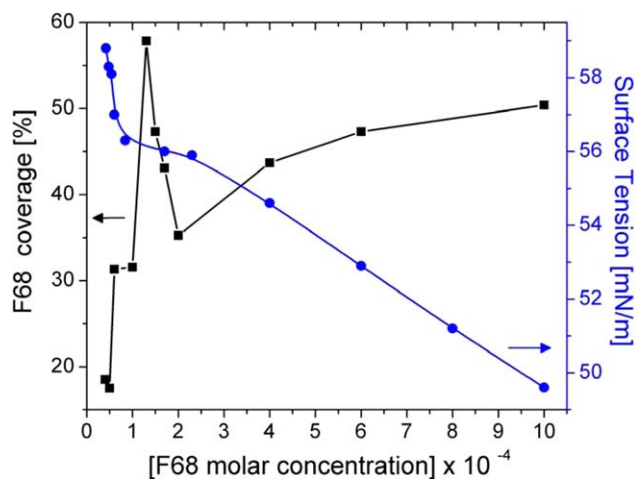


Figure 3. Percentage of F68 film coverage on silicon substrate as a function of the molar concentration of F68 as derived from AFM data (left), compared to the surface tension presented in Figure 1(b) (right). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

4(a). Below the values of the surface tension measured in the CMC range [Figure 1(b)], both variables presented a quadratic relationship ($R^2 = 0.9994$) as a result of the complete formation of micellar aggregates.³¹ A peak in the cells viability was observed at 56 mN/m, and above this value the viability rapidly decreases probably because of the high abundance of single block copolymer (unimers) and micelles aggregates.

CONCLUSIONS

From the analysis of our results, we conclude that in the CMC range, Pluronic F68 was capable to increase the viability of cells maintained under culture conditions. It suggested in the

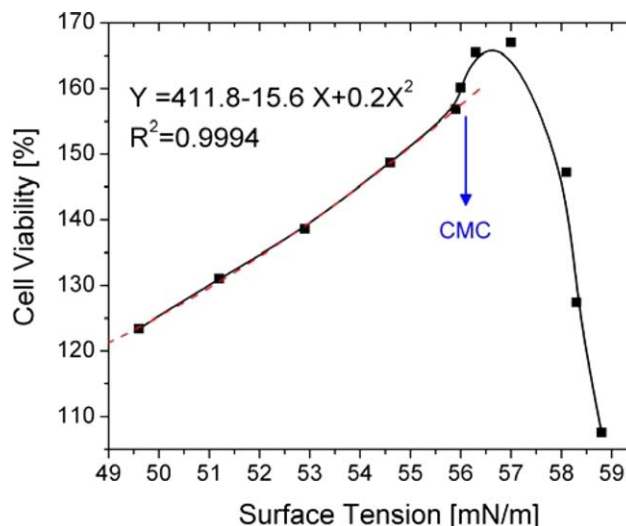


Figure 5. Cell viability in function of the surface tension, extracted from the data shown in Figures 3 and 4. The surface tension was constant during the CMC range (Figure 1), and produced a fast increase in the cells viability. Below this value, both variables presented a quadratic dependency. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

literature that the crucial role of unimers and micelles is determined by their ability to incorporate and translocate across the cell membranes.³² At low concentrations, the hydrophobic PPO moiety of F68 immerses into the membrane hydrophobic areas and alters the structure of the membrane, decreases microviscosity, and increases the surface tension.³³ This effect is observed below $1.2 \times 10^{-4} M$ in the surface tension [Figure 1(b)]. In the CMC range, the unimers aggregate in supramolecular structures, which affect the tension energy and the cell viability [Figure 4(b)]. Both present an inflection as a function of the F68 concentration. This transition is also consistent with the appearance of dendritic structures observed by AFM and the

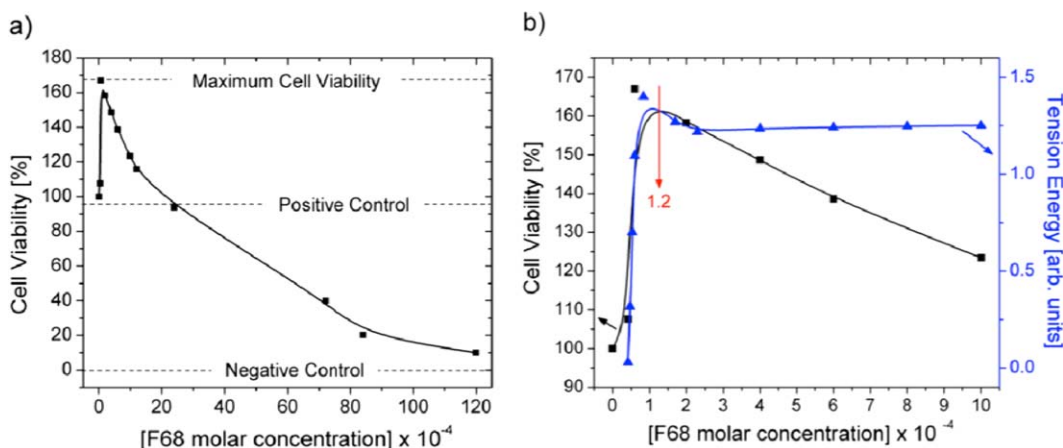


Figure 4. (a) Cell viability as a function of molar concentration of F68 in the culture medium of human neuroblastoma (SH-SYS5Y) differentiated with retinoic acid for two weeks. “Positive Control” refers to the cell culture before the inclusion of Pluronic F68 to the culture, and “Negative Control” refers to medium culture with all dead cells as described in the method section. (b) Cell viability and tension energy estimated from surface tension and AFM coverage data (Figure 3), as a function of F68 concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

increase of F68 coverage. At concentrations higher than $3.5 \times 10^{-4} M$ and lower than $20 \times 10^{-4} M$, the formation of micelles hides these hydrophobic PPO chains into the micellar core and thus diminishes the ability to protect the cellular membranes, in consistency with the report of Batrakova et al.³³ Above concentrations higher than $20 \times 10^{-4} M$, the cell viability decreases below 100% [Figure 4(a)] and, the cytotoxic effect of F68 is observed.

We finally conclude that this study demonstrates the close correlation between the dynamics of F68 micellization and its physicochemical properties, and highlights the importance of understanding the interactions between the cells and poloxamers as unimers and/or supramolecular aggregates in order to achieve a more controlled approach in their use as healing agents.

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