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Rheological properties of regular insulin and aspart insulin Langmuir monolayers at the air/water interface: Condensing effect of Zn^{2+} in the subphase

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ABSTRACT

The interfacial behavior of regular insulin (Reg-insulin) and aspart insulin (Asp-insulin) was critically affected by the presence of Zn^{2+} in the subphase. This cation induced a condensed-like behavior in the compression isotherms, especially apparent for Reg-insulin films when observed by Brewster angle microscopy. Immediately after spreading, Reg-insulin, but not Asp-insulin, showed bright patches that moved in a gaseous-like state. Moreover, Zn^{2+} caused marked variations of the surface electrostatics of both insulin monolayers and considerable hysteresis of their molecular organization. By oscillatory compression–expansion cycles, we observed in all cases the development of a dilatational response to the surface perturbation, and both monolayers exhibited well-defined shear moduli in the presence of Zn^{2+}, which was higher for Reg-insulin. Development of a shear modulus indicates behavior resembling a nominal solid, more apparent for Reg-insulin than for Asp-insulin, suggesting the presence of viscoelastic networks at the surface.

1. Introduction

Insulin is a polypeptide hormone (MW ~ 5800 Da) composed of two peptide chains linked by disulphide bridges differing in their hydrophobicity and charge at physiological pH [1,2]. Insulin is synthesized and stored in β cells of the pancreas as a biologically inactive Zn^{2+}-linked hexamer [3]. When released into the bloodstream, the hexamer dissociates into a dimer and subsequently into a monomer [4]. Insulin facilitates transport of glucose into muscle and fat cells in a process that involves at least two different steps: binding to a membrane receptor followed by activation of a glucose transporter [5–7]. The insulin monomer is less stable than the hexamer [8] and tends to aggregate [9]. An insulin analog with a diminished trend to aggregation is aspart insulin, in which a proline residue is substituted by aspartate in the B28 position, giving it an increased propensity to form monomers [10].

We recently showed that surfaces coated with insulin selectively influence hippocampal neuron polarization, depending on the molecular organization of the insulin film on which cells are grown [11]. The polarization pattern of neurons is also different than that induced through IGF-1 receptor activation by insulin added to the culture medium. This indicated that recognition events mediated by the neuronal membrane can finely differentiate the molecular organization of an insulin-coated surface [11]. This raises the question of whether other effects besides insulin–receptor interaction may respond to changes at the membrane surface. Insulin affects membrane viscoelastic properties at distances exceeding the effective diameter of the molecule, which suggested the long-range cooperative nature of insulin–membrane interaction [12]. Such cooperativity, and the concomitant reduction of membrane viscosity, may facilitate aggregation of insulin receptors, which is an important step for insulin action [13,14]. The significant changes induced by insulin on membrane viscoelasticity suggest that important biophysical factors, which would not consume energy, may underlie the action of the hormone [12,15,16].

Interfacial rheology is an important factor for the stability of emulsions and thin-liquid films containing proteins [17]. Proteins may stabilize interfaces by forming viscoelastic networks that can damp film-stretching and interfacial fluctuations, thus diminishing membrane-thinning [18]. These material properties can be characterized by dilatational and shear surface viscosities, and the respective elastic moduli [19].

Insulin Langmuir monolayers at the air/water interface were previously described under different experimental conditions such as pH, temperature and ion concentration [2,20,21]. Also, it has been recently shown that the presence of Zn^{2+} in the subphase has a profound effect on the surface behavior of insulin monolayers [20,21].

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As far as we know, comparative studies on the surface behavior of insulin and aspart insulin, their organization, electrostatics, surface topography and rheological properties, have not been reported. Considering the facts mentioned above, we first examined the surface organization of human insulin and aspart insulin monolayers at the air/water interface, with an emphasis on the viscoelastic properties of the films. Understanding the surface properties of insulin itself is of paramount importance and is an essential step for subsequent studies on its effects on the membrane structural dynamics. Scant information is available in this regard and will be the focus of the present work as a prior basic requirement for ensuing studies aimed at understanding the interaction of insulin with cell membrane lipids and proteins in reconstituted biomimetic surfaces with a controlled molecular organization for use as support for cellular growth.

2. Materials and methods

2.1. Reagents

Human regular insulin (Reg-insulin, Humulin R®, MW 5806) and human aspart insulin (Asp-insulin, NovoRapid Penfill Aspart Insulin®, MW 5826) were acquired from Eli Lilly and Co. (U.S.A.) and Novo Nordisk (Denmark), respectively. Insulins were further purified by size exclusion chromatography, liophilized and dissolved in ultrapure water with 0.06 M HCl, and then kept under refrigeration at 4 °C until use. Subphases were prepared with ultrapure water (resistivity 18.2 MΩ cm, Millipore water). NaCl and $ZnCl_2$ were purchased from Merck (Darmstadt, Germany).

3. Insulin monolayers

3.1. Compression isotherms

The absence of surface-active impurities was checked before spreading the monolayers or in spreading solutions, as described elsewhere [22]. Insulin Langmuir monolayers were formed by spreading 6 μL of insulin solutions (3.5 mg/mL) onto the aqueous surface (NaCl 145 mM, pH 5.6 or NaCl 145 mM plus $ZnCl_2$ 1 mM, pH 6.3). Before film compression 10 min was allowed for monolayer equilibration. Compression–expansion isotherms were carried out with two symmetrically moving barriers in a KSV-minitrough (KSV Instruments, Helsinki, Finland). Reducing the barrier speed by half did not affect the isotherms. The temperature of the subphase was maintained at 24 °C ± 0.5 with an external circulating water bath (Haake F3C). Collapse and surface pressure (Π) points for molecular reorganization and limiting mean molecular areas (MMA) were determined from the third derivative of the compression isotherms [23].

3.2. Surface potential (ΔV)

ΔV measurements were performed with a vibrating plate (KSV Instruments, Helsinki, Finland) enclosed in an acrylic box surrounded by a grounded metallic grid. The resultant perpendicular dipole moment of insulin is directly proportional to the surface (dipole) potential per unit of molecular surface density $[\Delta V/n = \Delta V{\cdot}\mathrm{MMA}]_{\Pi}$ where n is the density of overall molecular dipoles in the film (the inverse of MMA) at a defined Π [24].

3.3. Brewster angle microscopy (BAM)

For film imaging, BAM was performed with an autonulling imaging ellipsometer (Nanofilm EP3SE, Accurion GmbH, Germany) equipped with a 532 nm laser, 10× and 20× objectives and a CCD camera. By using p-polarized light, the equipment automatically measures the intensity of reflected light at several angles of incidence and performs a calibration of the reflected light and the gray level intensity (reflectivity, $R = I/I_0$, where I is the reflected light intensity and I_0 is the incoming beam intensity).

3.4. Thermodynamic functions of hysteresis

In ideally fluid-films, hysteresis is absent, $\Delta G_i^{\mathrm{hys}} = 0$, $\Delta S_i^{\mathrm{hys}} = 0$ and $\Delta H_i^{\mathrm{hys}} = 0$. The free energy of hysteresis ΔG^{hys}, the configurational entropy of hysteresis ΔS^{hys} and the enthalpy of hysteresis ΔH^{hys} are defined by Eqs. (1)–(4), respectively [25]:

$$\Delta G^{\mathrm{hys}} = \Delta G_{\mathrm{expan}} - \Delta G_{\mathrm{comp}} \tag{1}$$

$$\left[\Delta S_{\prod}^{\mathrm{hys}} = R \ln \frac{\mathrm{MMA}_{\mathrm{expan}}}{\mathrm{MMA}_{\mathrm{comp}}}\right]_{\prod} \tag{2}$$

$$\Delta S^{\mathrm{hys}} = \sum\nolimits_{\prod} \Delta S_{\prod}^{\mathrm{hys}} \tag{3}$$

$$\Delta H^{\mathrm{hys}} = \Delta G^{\mathrm{hys}} + T\,\Delta S^{\mathrm{hys}} \tag{4}$$

For the thermodynamic functions of hysteresis, the difference is taken between the values of expansion and those of compression. Hysteresis cycles showed negligible variation after two successive compression–expansions, and reducing the barrier speed by half did not introduce significant changes (not shown).

4. Rheology measurements

4.1. Surface compressional modulus (K^s)

K^s (in-plane elasticity) [23,24] was calculated directly from the isotherms according to

$$K^s = -\mathrm{MMA}\left(\frac{\partial \prod}{\partial \mathrm{MMA}}\right)_T \tag{5}$$

4.2. Dilatational and shear moduli

Insulin monolayers were compressed isometrically up to the desired Π. Then, sinusoidal area perturbations were carried out while simultaneously measuring both Π and phase-shift in response to area changes [19].

Since the deformation created by the moving barriers is uniaxial, it contains a superposition of well-defined dilatation and shear. Thus, as the interfacial pressure is a tensor quantity, the values depend on the direction along which the stress is acting [17]. Therefore, if two Wilhelmy plates are placed in orthogonal positions, the following equations describe the total viscoelastic response of the system:

$$\left|\varepsilon + G\right| = A_0 \frac{\Delta \prod_{\parallel}}{\Delta A} \tag{6}$$

$$\left|\varepsilon - G\right| = \mathrm{A}_0 \frac{\Delta \prod_{\parallel}}{\Delta \mathrm{A}} \tag{7}$$

where ε is the (complex) dilatational compressibility, G is the (complex) shear modulus and $\Delta\Pi_{\parallel}$ and $\Delta\Pi_{\perp}$ are the lateral pressure response registered by the sensing plates placed parallel and perpendicular to the barriers, respectively. If the layer is purely elastic,

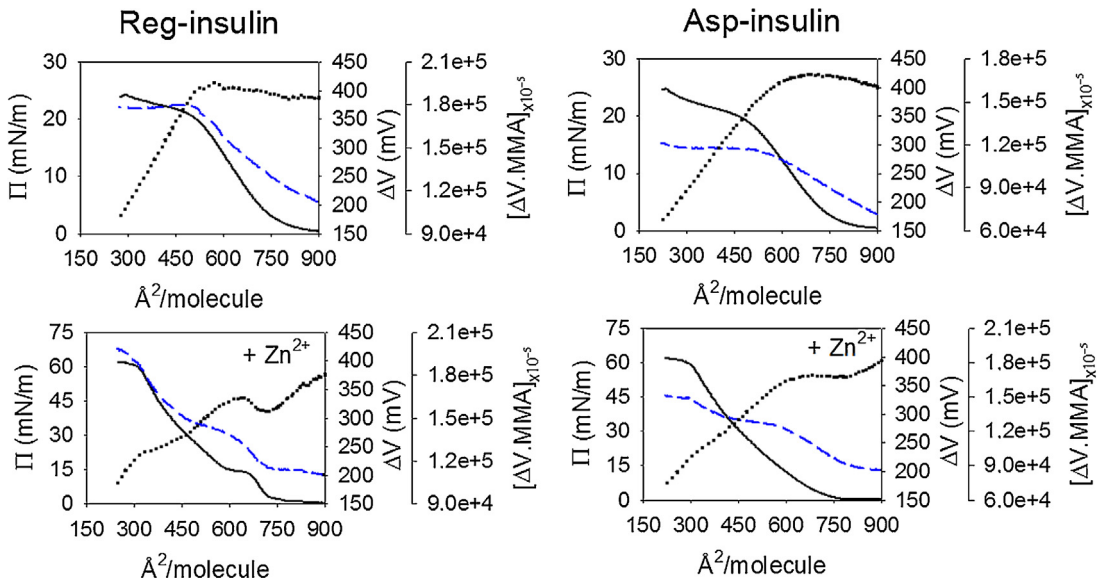

Fig. 1. Surface behavior of insulin Langmuir monolayers. Surface pressure (Π, continuous line), surface potential (ΔV, dashed line) and surface potential per unit of molecular surface density ([ΔV·MMA], dotted line), as a function of the mean molecular area (MMA), for Reg-insulin and Asp-insulin monolayers with or without Zn^{2+}. Compression speed: 10 mm/min.

then area and Π should always be in phase; if it is purely viscous, both parameters should be 90° out of phase [19]. Usually, the frequency dependent complex modulus $\varepsilon(\omega)$ is defined as:

$$\varepsilon(\omega) = \varepsilon'(\omega) + i\varepsilon''(\omega) = \varepsilon'(\omega) + i\omega\eta(\omega) \quad (8)$$

where $\varepsilon'(\omega)$ and $\omega\eta_d(\omega)$ are the dilatational elasticity and the dilatational viscosity, respectively. The real part is the elastic (storage) component of the modulus:

$$\varepsilon' = |\varepsilon| \cos\varphi \quad (9)$$

and the imaginary part is the dissipative (loss) component:

$$\varepsilon'' = |\varepsilon| \sin\varphi \quad (10)$$

The phase angle φ is equal to $2\pi\omega\delta t$.

The shear elastic modulus G is defined as the ratio between the increase of shear strain and shear stress; the shear viscosity η_s is the ratio between the shear stress and the rate of shear. In an oscillatory measurement one can measure the complex shear modulus as follows:

$$G(\omega) = G'(\omega) + iG''(\omega) = G'(\omega) + i\omega\eta(\omega) \quad (11)$$

Where the real part is the elastic (storage) component of the modulus:

$$G' = \left|G\right| \cos\varphi \quad (12)$$

and the imaginary part is the dissipative (loss) component:

$$G'' = \left|G\right| \sin\varphi \quad (13)$$

5. Results and discussion

5.1. Surface behavior of insulin monolayers

The Π and ΔV·MMA isotherms are shown in Fig. 1. The isotherm lift-off corresponds to a MMA of ~900 Å²/molecule for insulin films (regular and aspart) on NaCl 145 mM, pH 5.6–10, and ~800 Å²/molecule for insulin films (regular and aspart) on NaCl 145 mM plus 1 mM $ZnCl_2$, pH 6.3. On decreasing the MMA from 900 to ~625 Å²/molecule, a highly compressible state of both insulin films without Zn^{2+} was observed. From a MMA of ~530 Å²/molecule (18 mN/m), these monolayers reach the collapse at ~23 mN/m and ~470 Å²/molecule. In contrast, the Reg-insulin monolayer with Zn^{2+} becomes more condensed, under compression, between ~690 Å²/molecule (8 mN/m) and 630 Å²/molecule (14 mN/m). A Π-induced reorganization under compression was observed at 14 mN/m. Collapse was observed at Π ~ 55–60 mN/m and MMA ~ 300 Å²/molecule. By contrast, we observed that bovine Reg-insulin reorganizes at $\Pi \approx 18$ mN/m [11], in general agreement with Pérez-López et al. [20]. Nieto-Suárez et al. [2] showed that NaCl does not alter the shape of the isotherms (compared to films on pure water), but slightly increases the film area (for a given Π). They attributed this slight expansion to a salting out effect, where the ions may weaken the hydration of protein polar groups, thereby increasing repulsion between and within the insulin molecules. Our results also confirmed the findings of Nieto-Suárez et al. [2] that Zn^{2+} causes reduction of the area (lift-off) of uncompressed films, considerably increases the slope and area of the isotherm in pre- and post-transition regions and introduces a sharp slope change that appears to reflect a genuine collapse at Π = 55–60 mN/m and MMA = 200 Å²/molecule.

The collapse of Asp-insulin in the presence of Zn^{2+} also occurred at a higher Π (60 mN/m) and a more condensed MMA (~300 Å²/molecule) than without Zn^{2+}. However, Π-induced reorganization, clearly present in Reg-insulin, was not ostensible in compression Π-MMA isotherms of Asp-insulin.

Zn^{2+} caused marked variations of the surface electrostatics of insulin monolayers. The relatively high ΔV reached at close packing by Reg-insulin monolayers correlates with a more condensed organization. The variation of ΔV per unit of molecular surface density $[\Delta V/n = \Delta V{\cdot}\mathrm{MMA}]_\Pi$ is directly proportional to the packing and changes in organization of the average resultant molecular dipole moment perpendicular to the interface under compression. Several combined electrostatic contributions, which cannot be readily separated, are involved in this parameter [23]. With Zn^{2+}, the variation of ΔV for both insulins at low and high Π (Fig. 1 and Supplementary Fig. 1) shows that Asp-insulin, similarly to Reg-insulin, undergoes a reorganization of surface dipoles at $\Pi \approx 15$ mN/m.

The condensed-like behavior induced by Zn^{2+} was especially apparent in Reg-insulin when observed by BAM. As shown in Supplementary Fig. 2, immediately after spreading we observed insulin patches moving within a gaseous state. The increased reflectivity

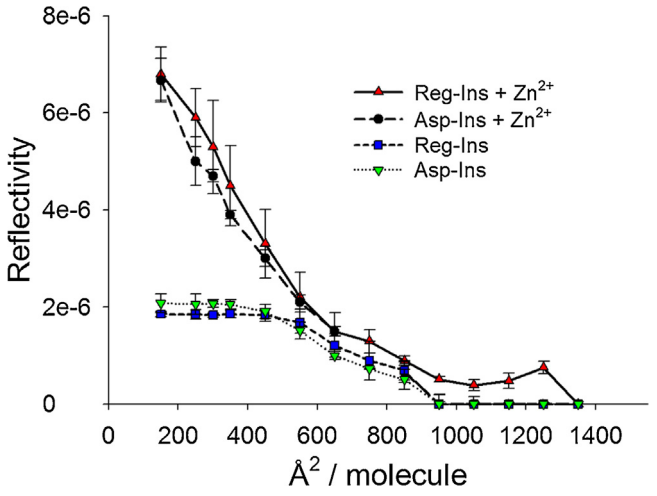

Fig. 2. Reflectivity of insulin monolayers. ▲: Reg-insulin + Zn^{2+}, ●: Asp-insulin + Zn^{2+}, ■: Reg-insulin, ▼: Asp-insulin. Symbols correspond to average values ± SEM of three independent experiments.

(Fig. 2) in both insulin monolayers as they become more condensed matches the behavior in the compression isotherms. For several lipid and protein interfaces it is known that condensed states exhibit increased reflectivity due to increased interfacial thickness, variation of its refraction index, or both [26].

The more condensed behavior of Reg-insulin with Zn^{2+} may be related to the formation of hexamers [8,27]. Nieto-Suárez et al. [2] proposed that Zn^{2+} not only reduces the area of uncompressed Reg-insulin films, but also make the film behave as a genuine monolayer of hexamers (with defined Le-like and Lc-like regions, and a clear collapse) by preventing the presence of monomers and the partial immersion of the hydrophilic A chains in the subphase. The reorganization observed for Reg-insulin in the presence of Zn^{2+} may be ascribed to a reorientation of hexamers at the interface [2]. Evidence suggesting the formation of hexamers in Reg-insulin monolayers was also provided by Liu et al. [21] by infrared reflection-absorption spectroscopy (IRRAS). It was deduced that an α-helix conformation was mostly maintained in the monolayer [21,28], and hexamer formation could occur by an increase of β-strand structures [21]. The reorientation of Reg-insulin molecules at the interface involves different orientation of the A or B chains in a pH-dependent manner [21]. However, without Zn^{2+}, the Reg-insulin monolayer was not aggregated and had an α-helix conformation over the whole isotherm [28]. These facts are well correlated with bulk determinations of insulin secondary structure either for hexamers or monomers [29].

Reg-insulin and Asp-insulin show a high tendency to adsorb to hydrophobic–hydrophilic interfaces such as Teflon-water and air/water [2,11,21,30]. After adsorption, conformational changes in both insulins were described by circular dichroism (CD) and differential scanning calorimetry (DSC). The secondary structure was modified in the adsorbed state, both insulins losing α-helical to random coil structures [30]. Mollman et al. [30] proposed that changes exposing hydrophobic domains, probably located in the conformation of the C-terminal part of the B-chain, were involved in the adsorbed states.

A single substitution at residue B28 is sufficient to give Asp-insulin a markedly increased tendency to form monomeric units upon dilution. Mutation of B28 is significant, because this residue makes van der Waals contacts with residues B20–B23 of the adjacent monomer [31,32], and the hexamer formation of Asp-insulin in the presence of Zn^{2+} is critically diminished [33]. Our findings are consistent with this fact. Compared to Reg-insulin, Π-induced reorganization of Asp-insulin films with Zn^{2+} goes undetected in the Π-MMA compression isotherm. Moreover the Reg-insulin-condensed clusters formed immediately after spreading were not observed in the Asp-insulin gaseous-like state (Π < 1 mN/m) (Supplementary Fig. 2).

The Π-dependent variation of ΔV·MMA of both types of insulin indicates that, without Zn^{2+}, their overall resultant moment perpendicular to the interface remains approximately invariant under compression until collapse. On the other hand, with Zn^{2+} in the subphase, ΔV·MMA decreases throughout the compression isotherm for both Reg-insulin and Asp-insulin, revealing that dipolar reorganization occurs in the latter at ~15–20 mN/m, despite not being detected in the Π-MMA compression isotherm. This Zn^{2+}-induced reorganization becomes emphasized under film expansion (Fig. 3 and Supplementary Fig. 1), which clearly reflects the molecular reorganization taking place under increasing molecular packing This occurs with dipolar contributions that contain increasingly positive electrostatic components pointing toward the subphase, thus reducing the overall ΔV per unit of molecular surface density. The changes under expansion (Supplementary Fig. 1) indicate that, once a defined molecular organization is acquired at high Π, the dipolar arrangements remain mostly invariant over a relatively large range of area expansion, thus exhibiting electrostatic hysteresis as well.

The marked effect of Zn^{2+} on the properties of insulin monolayers is in keeping with the influence of other divalent cations on biointerfaces. Ca^{2+} modified the interaction of pulmonary surfactant protein SP-A with monolayers of DPPC:PG through their effects on the conformation, self-association and charge state of SP-A [34]. Mg^{2+}, Ca^{2+} and Sr^{2+} interact differently with a DMPG monolayer in pure water at pH 7. In the presence of 1 mM $CaCl_2$, a condensation of the DMPG film is induced, while an expansion of the monolayer is observed in the presence of Mg^{2+} and Sr^{2+} [35]. The molecular packing and ΔV of monolayers formed by zwitterionic phospholipids and several mixtures of fatty acids, glycerides and glycosphingolipids are modified by uranyl ions and Ca^{2+} in the aqueous medium [36,37].

5.2. Hysteresis of insulin monolayers

The occurrence of hysteresis under film compression–expansion is a common phenomenon in Langmuir monolayers [25]. These effects arise from a balance among cohesion phenomena and viscoelastic properties of the interface that have different reversibility properties. Since the viscous (dissipation) are lower than the elastic components in the film (see below), we consider that hysteresis is likely related to the stability of closely packed states formed under compression, and their tendency to slowly undergo reversible reorganization to their initial state under expansion [38]. This assumption is also supported by the relative invariance of the interfacial electrostatics (reflected by ΔV·MMA) up to increased MMA under expansion after compression to close packing (see above). Such molecular arrangements also depend on differences in the energy and kinetic processes required for intermolecular cohesion and dispersion upon compression/expansion [25].

Fig. 3 shows hysteresis cycles of the monolayers at two Π_{target} (15 and 50 mN/m). In the hysteresis cycle, the ΔG^{hys} of Reg-insulin with Zn^{2+} at Π_{target} = 15 mN/m is higher than that found for both Reg-insulin without Zn^{2+} and Asp-insulin irrespective of the presence of Zn^{2+} (Table 1). The negative values of ΔG^{hys} indicate the retention of a considerable amount of free energy in the cycle, where Zn^{2+} adds a substantial element to facilitate and retain cohesive interactions among film molecules. By contrast, the values of thermodynamic functions of hysteresis are quite similar for Reg-insulin and Asp-insulin on subphases with Zn^{2+} at Π_{target} = 50 mN/m, despite the differences observed in their

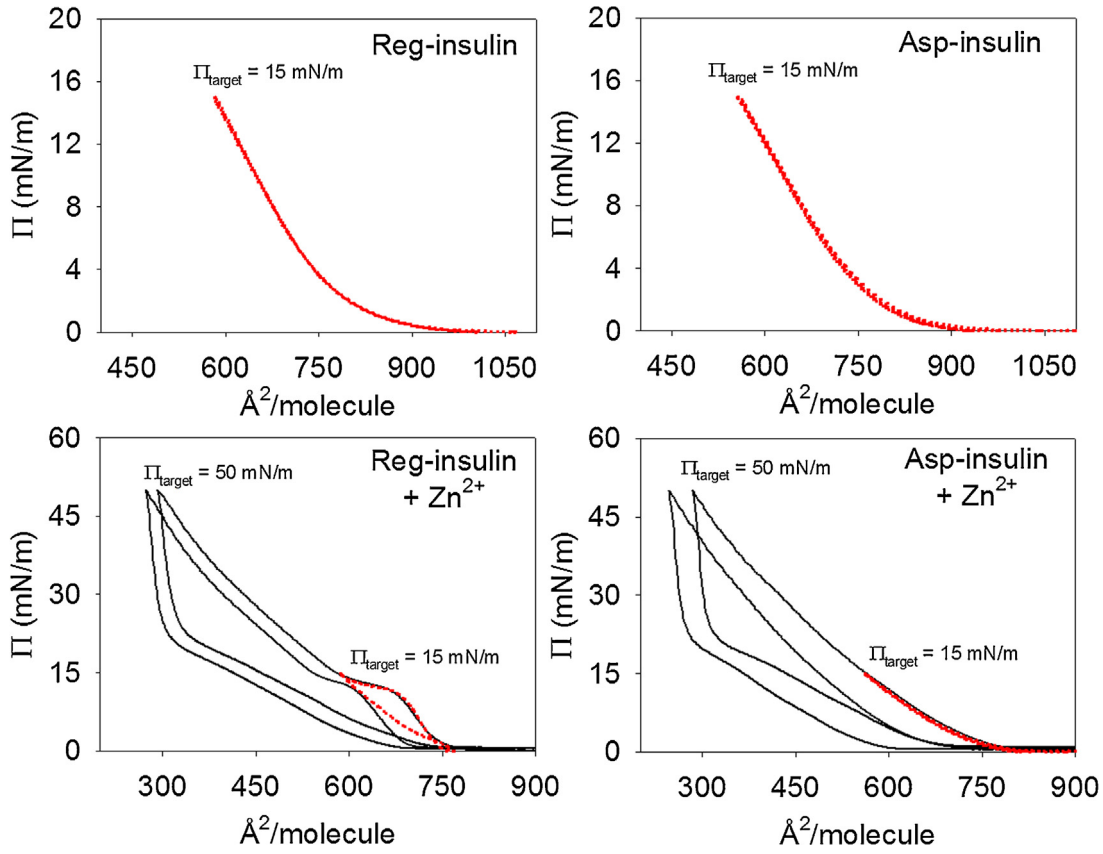

Fig. 3. Hysteresis of Reg-insulin and Asp-insulin monolayers with or without Zn^{2+}. Compression–expansion cycles were performed at 24 °C (speed: 10 mm/min) and at two desired surface pressures (Π_{target}). Continuous lines: Π_{target} = 50 mN/m. Dashed lines: Π_{target} = 15 mN/m.

compression isotherms and interfacial topography revealed by BAM. This would be possible only if interactions established during the compression of the monolayers were of similar energy.

Fig. 4 shows the ΔG^{hys} values of insulins in the presence of Zn^{2+} as a function of Π_{target}. Hysteresis of Reg-insulin becomes apparent at a relatively low Π (15 mN/m) compared to Asp-insulin in which no hysteresis is observed up to 20 mN/m. Nevertheless, ΔG^{hys} values of Reg-insulin up to 15 mN/m and of Asp-insulin up to 20 mN/m, both in the presence of Zn^{2+}, are small (Reg-insulin) or negligible (Asp-insulin) compared to the changes of ΔG^{hys} when the films were compressed to higher Π. This means that relatively long-lived cohesive interactions become stabilized after the molecular reorganization under compression surpasses a threshold in the range of 15–20 mN/m; in Asp-insulin, the reorganization is not apparent in the Π-MMA isotherm, probably due to rather poor cooperativity for molecular cohesion, but can be clearly detected by the variation of the more sensitive ΔV·MMA.

The relatively large negative ΔS^{hys} observed in both insulin monolayers with Zn^{2+} at Π_{target} = 50 mN/m indicates the formation of similar entropically unfavorable, more compact and ordered molecular organizations. Such states can be adopted under compression by the establishment of enthalpically favorable (exothermic) interactions, as revealed by the considerable negative enthalpy of hysteresis calculated from the observed negative ΔG^{hys} and ΔS^{hys}. Interestingly, the Π-MMA isotherms of Asp-insulin films (with Zn^{2+} at Π_{target} = 50 mN/m) under expansion revealed

Table 1
Thermodynamic functions of hysteresis.

	ΔG_{comp} (kcal mol^{-1})	ΔG_{expan} (kcal mol^{-1})	ΔG^{hys} (kcal mol^{-1})	ΔS^{hys} (kcal mol^{-1} K^{-1})	ΔH^{hys} (kcal mol^{-1})
Π_{target} = 15 mN/m					
Reg-Ins 1st cycle	2.29 ± 0.4	2.26 ± 0.2	−0.04 ± 0.01	−0.073	−0.041
Reg-Ins 2nd cycle	2.27 ± 0.1	2.22 ± 0.2	−0.05 ± 0.02	−0.059	−0.042
Asp-Ins 1st cycle	2.48 ± 0.2	2.44 ± 0.1	−0.04 ± 0.01	−0.096	−0.048
Asp-Ins 2nd cycle	2.43 ± 0.1	2.37 ± 0.1	−0.06 ± 0.02	−0.078	−0.052
Reg-Ins Zn 1st cycle	2.42 ± 0.5	1.68 ± 0.4	−0.74 ± 0.21	−0.633	−0.556
Reg-Ins Zn 2nd cycle	2.27 ± 0.3	1.62 ± 0.3	−0.65 ± 0.14	−0.632	−0.719
Asp-Ins Zn 1st cycle	2.06 ± 0.4	2.01 ± 0.2	−0.05 ± 0.01	−0.047	−0.0.41
Asp-Ins Zn 2nd cycle	1.92 ± 0.3	1.85 ± 0.3	−0.07 ± 0.01	−0.051	−0.049
Π_{target} = 50 mN/m					
Reg-Ins Zn 1st cycle	15.38 ± 2.0	8.21 ± 1.0	−7.17 ± 0.9	−21.276	−9.90
Reg-Ins Zn 2nd cycle	14.08 ± 1.3	7.13 ± 0.5	−6.95 ± 0.5	−22.005	−10.09
Asp-Ins Zn 1st cycle	14.73 ± 1.4	3.08 ± 0.4	−11.65 ± 1.8	−22.018	−12.36
Asp-Ins Zn 2nd cycle	12.88 ± 1.7	4.79 ± 0.6	−8.09 ± 1.5	−23.300	−10.97

The free energy of compression (ΔG_{comp}), expansion (ΔG_{expan}), hysteresis (ΔG^{hys}), the configurational entropy of hysteresis (ΔS^{hys}) and the enthalpy of hysteresis (ΔH^{hys}) calculated between V = 2–15 mN/m and 2–50 mN/m, for insulins in absence and presence of Zn^{2+}, respectively. All experiments were performed by triplicate ± SEM.

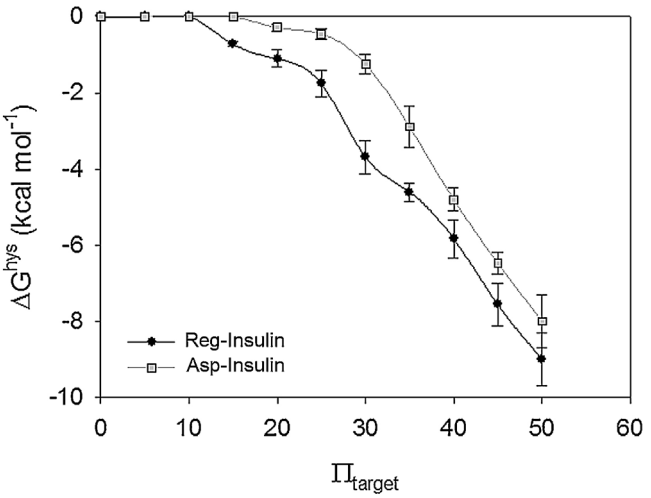

Fig. 4. Hysteresis as a function of target surface pressure. We determined the partial ΔG^{hys}, of regular and aspart insulins in presence of Zn^{2+}, by performing hysteresis cycles at a desired surface pressure (Π_{target}). Average values ± SEM are result of four independent experiments.

a similar molecular reorganization to that found for Reg-insulin occurring at ~21–28 mN/m and 350–500 Å^2/molecule (Fig. 3). This suggests, as mentioned in the previous section for the ΔV, that a similar surface organization was reached in both insulin films when compressed to high Π.

5.3. Rheology of insulin monolayers

Fig. 5 shows the isothermal K^s for Reg-insulin and Asp-insulin, with and without Zn^{2+}, calculated by Eq. (5). As shown in the figure, the slope changes of compression isotherms (Fig. 1) were clearly emphasized by the K^s values. For Reg-insulin, with Zn^{2+}, we observed a larger K^s at a MMA of ~700 Å^2/molecule, indicating the attainment of a more condensed state than that of the Asp-insulin monolayer at a similar molecular packing. The decrease of K^s value at 625 Å^2/molecule of Reg-insulin monolayers with Zn^{2+} reflects the change of in-plane elasticity occurring at ~15 mN/m (Fig. 1).

The response to deformation by compression of an isotropic 2D material is characterized in general by two elastic moduli: changes in area are controlled by the compressional viscoelastic modulus ε (also referred to as dilatational modulus), and changes in shape by the shear modulus G [19]. K^s is equal to ε only if the rate of compression is quasi-static. However, the experimentally available compression velocities are usually too fast for obtaining a quasi-static compression. Thus, considering this experimental limitation, a quite simple method for studying the complex dilatational modulus is to perform oscillatory compression–expansion cycles of the film at a certain frequency and measure the Π and phase-shift related to surface area signals [19]. By these uniaxial compressions, the film is actually subjected to both compression and shear. Moreover, the real and imaginary parts of the moduli can be discriminated [19].

Fig. 6 illustrates an example of typical oscillatory measurements. The Π is recorded as a function of time, while the area is changed by imposed oscillatory barrier movements at a defined frequency (10 mHz). We held the amplitude oscillation at a constant value (3% area change). Martin et al. [39] showed that in adsorbed β-lactoglobulin films the response is linear up to 4%. However, our linear response was only up to 3% (Supplementary Fig. 3). In a typical experiment, data points are recorded every 0.4 s (our time resolution was 0.34 ± 0.04 s). The data are then analyzed by finding the set of times at which the area value is A_0 and the set of times at which the pressures are Π_0. Then the value of δt is calculated as

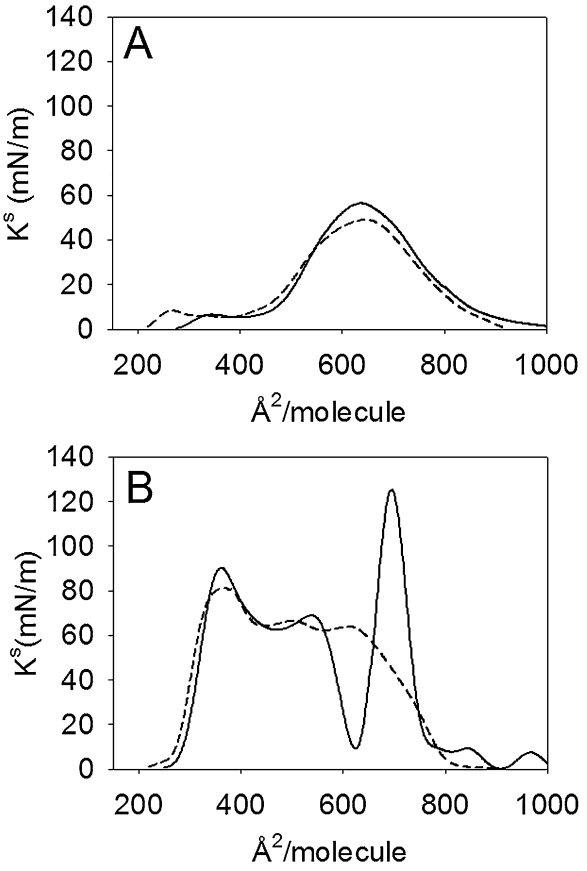

Fig. 5. Surface compressional moduli, K^s. (A) Reg-insulin (continuous line) and Asp-insulin (dashed line) in absence of Zn^{2+}. (B) Idem for A but in presence of Zn^{2+}.

the average of the difference between each of the time-set values, acquired for orthogonal and parallel orientations of the barriers, from which two values of $\delta_\perp$ and $\delta_\parallel$ are obtained. The Supplementary Table 1 shows the average δt as a function of surface pressure with or without Zn^{2+}. Reg-insulin with Zn^{2+} shows an average δt significant to $p \leq 0.02$ in all cases when compared to Reg-insulin without Zn^{2+}. This was also true for Asp-insulin with Zn^{2+} but at a higher surface pressure (15 mN/m).

Our equipment noise is ~0.1 mN/m at a temperature of 24 °C and average reproducibility of surface pressure–area compression isotherms is generally about 1 mN/m. We calculated the statistical absolute difference of the Π-change for both insulins at a defined surface pressure (used for the oscillatory experiments) with or without Zn^{2+} (see Supplementary Table 2). Thus we assume that a shear modulus is developed when the absolute difference of the Π-change is at least 1 mN/m and statistically significant. In Fig. 6, only a small difference between $\Pi_\parallel$ and $\Pi_\perp$ of Reg-insulin and Asp-insulin without Zn^{2+} was observed. On the other hand, with Zn^{2+}, the oscillatory response around 5 mN/m becomes clearly anisotropic ($\Pi_\parallel > \Pi_\perp$) in Reg-insulin films. This indicates the existence of a shear modulus. This is also true for Asp-insulin with Zn^{2+} at higher pressures.

The values of ΔA and $\Delta\Pi$ are extracted from the data traces by calculating the average of the difference between the maximum and minimum values. From these values we calculate $|\varepsilon + G|$ and $|\varepsilon - G|$ from Eqs. (6) and (7) (see Fig. 7C and D). The phase angle φ and the elastic and dissipative components of the response are obtained from Eqs. (9)–(13).

Fig. 7A and B shows the frequency dependence, at $\Pi_0 = 5$ mN/m, of the four components of the total viscoelastic response obtained by Eqs. (6) and (7). We observed no differences between $|\varepsilon + G|$

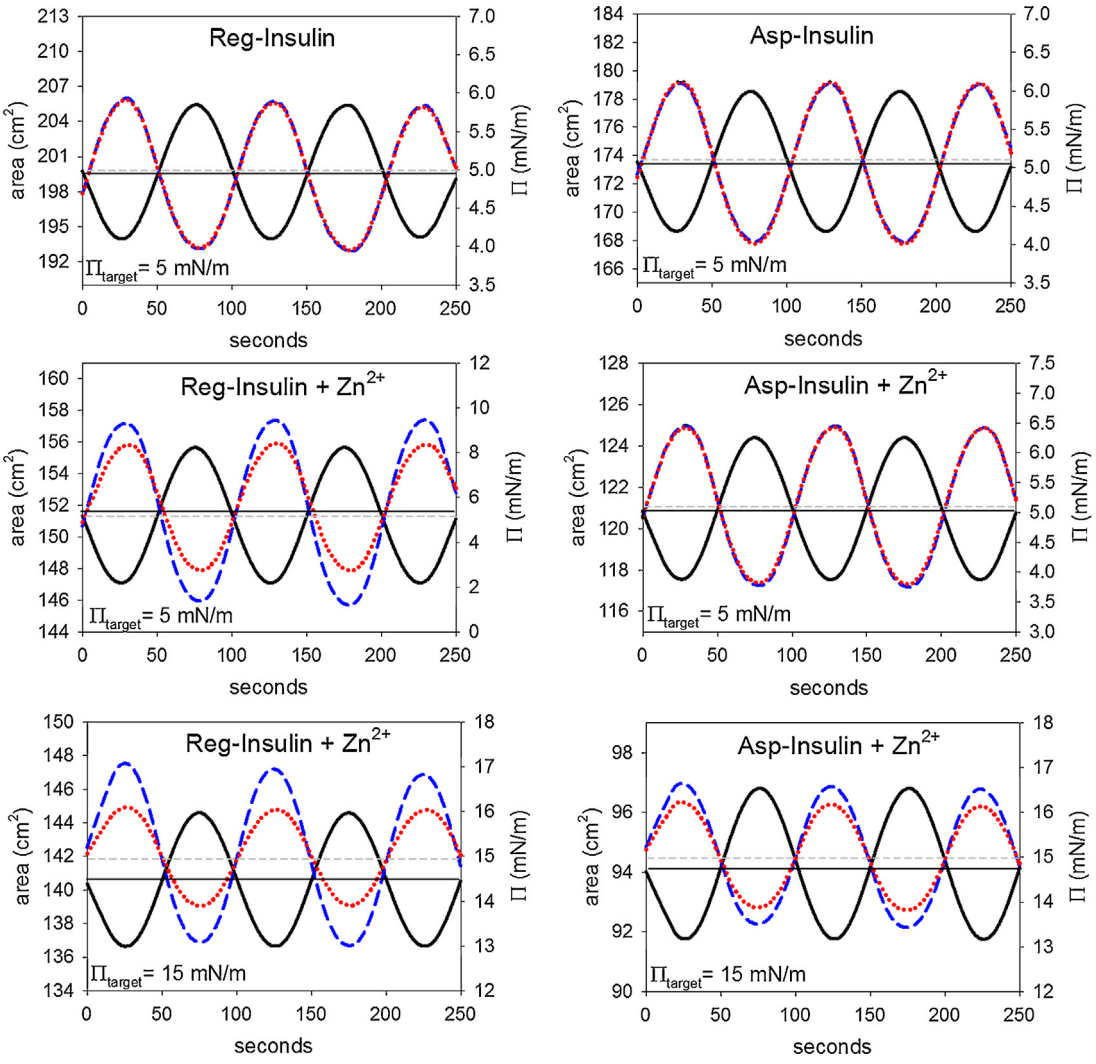

Fig. 6. Barrier oscillation experiment. Compression–expansion cycles of both insulin monolayers, with and without Zn^{2+} at Π_0 = 5 mN/m and Π_0 = 15 mN/m for insulins with Zn^{2+}. All oscillatory experiments were performed at a frequency of 10 mHz and 3% of amplitude. Continuous lines correspond to area change. $\Pi_{\parallel}$ (dashed lines) and $\Pi_{\perp}$ (dotted lines) correspond to parallel and perpendicular plate orientations. Horizontal continuous and dashed lines correspond to A_0 and Π_0, respectively.

and $|\varepsilon - G|$ of both insulin films on subphases without Zn^{2+} whereas, with Zn^{2+}, Reg-insulin showed $|\varepsilon + G| > |\varepsilon - G|$ but only at 10 mHz. Thus all subsequent oscillatory experiments were performed at 10 mHz and 3% area-change (amplitude). Similar to a study performed by Hilles et al. [40], we show in Supplementary Fig. 4 that in Reg-insulin monolayers with Zn^{2+} there is no hysteresis up to a Π_{target} = 10 mN/m. At 15 mN/m, we observed a small hysteresis involving about 0.67 kcal mol^{-1}, as shown in Fig. 4. Asp-insulin monolayers with Zn^{2+} did not show hysteresis up to 20 mN/m. Also, Reg-insulin and Asp-insulin monolayers without Zn^{2+} showed no significant hysteresis up to 15 mN/m. Thus all oscillatory experiments were developed at Π where small or negligible hysteresis was present.

Fig. 8A shows the dilatational and shear modulus as a function of Π. The elastic response ε' of monolayers formed by Reg-insulin and Asp-insulin without Zn^{2+} is smaller than ε' of Reg-insulin films with Zn^{2+}, reaching a maximum value of ~40 mN/m. In contrast, the maximum value of ε' (~90 mN/m) was observed for Reg-insulin at Π = 5 mN/m with Zn^{2+}. At Π = 15 mN/m we observed a relative decrease of ε' for Reg-insulin, corresponding to the Le and Lc-like transition. However, in the case of Asp-insulin with Zn^{2+}, the dilatational elastic response ε' increases smoothly as a function of Π. The dilatational viscous response, represented by the loss modulus ε'', was smaller in all cases, indicating that the dilatational response of insulin monolayers was mainly elastic. Nevertheless, a larger viscous response was observed for Reg-insulin in the presence of Zn^{2+} at 5 mN/m. The K^s modulus of Le and Lc-like states (Π range = 0–35 mN/m) for Reg-insulin and Asp-insulin in the absence and presence of Zn^{2+} is shown in Fig. 8B. Note that the dilatational moduli are well-correlated to the K^s values, except for Reg-insulin with Zn^{2+} at 15 mN/m where the value of the latter is smaller. The increase, combined with the decrease observed in K^s of Reg-insulin with Zn^{2+}, is a consequence of the molecular reorganization taking place in the compression isotherm (plateau). It is important to note that K^s contains information only about elasticity [23,24]. The similarity observed between K^s and ε' is because the latter is a combination of compressibility and dilatational rheology [19]. Thus, we conclude that the elastic response ε is strongly dominated by the compressibility (see the higher values of ε' compared to ε'').

We found that a shear viscoelastic response developed only in insulin monolayers with Zn^{2+}. Reg-insulin showed the largest elastic response G' of the shear modulus, starting from Π = 5 mN/m and increasing continuously, reaching a G' value of ~11 mN/m at Π = 15 mN/m. Asp-insulin developed a shear elastic response G' of 1.5 mN/m from Π = 15 mN/m to a maximum value of 7.5 mN/m at Π = 20 mN/m. Similar to the dilatational modulus, the shear viscous

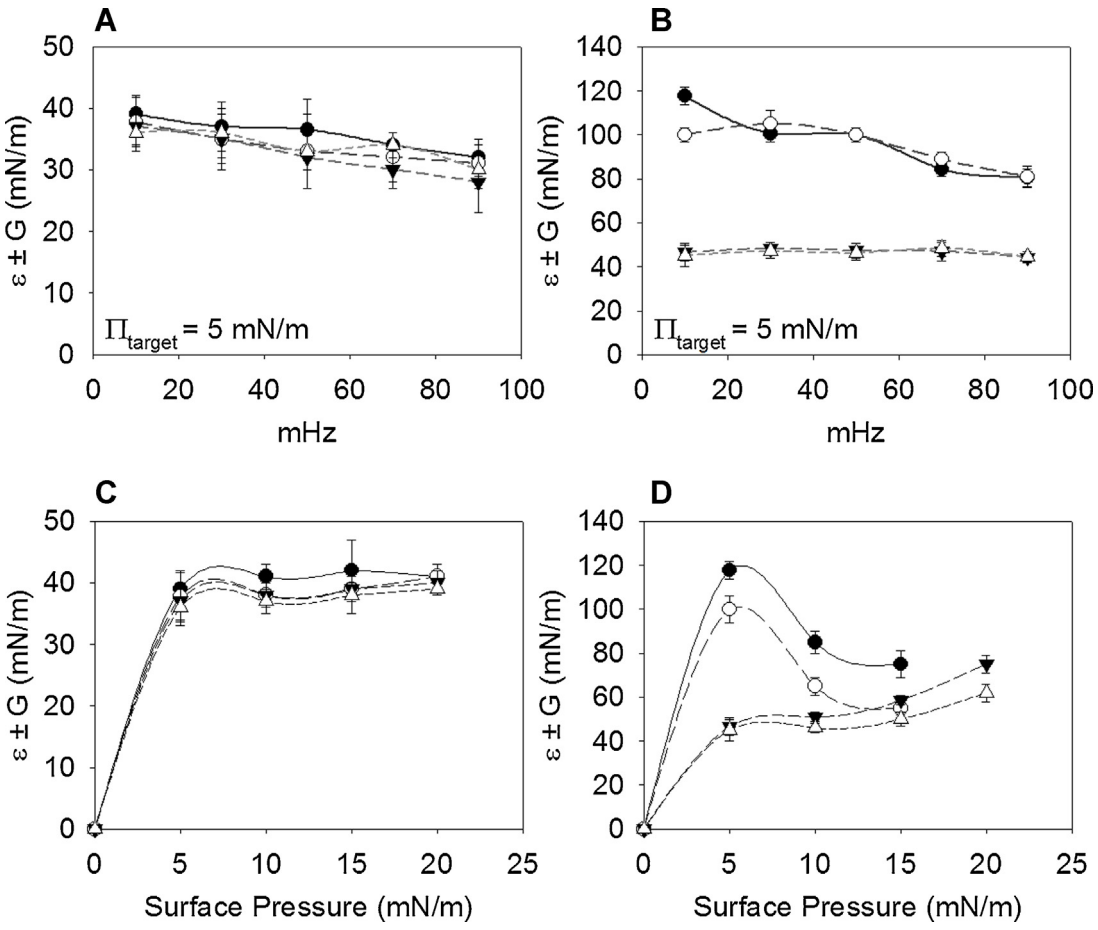

Fig. 7. Total viscoelastic response of insulin monolayers. (A) and (B) Reg-insulin $\varepsilon + G$ (●) and $\varepsilon - G$ (○) and Asp-insulin $\varepsilon + G$ (▼) and $\varepsilon - G$ (△) as a function of frequency without and with Zn^{2+}, respectively. Oscillatory experiments were performed at a Π_{target} = 5 mN/m and 3% amplitude. (C) and (D) Reg-insulin $\varepsilon \pm G$ and Asp-insulin $\varepsilon \pm G$ as a function of surface pressure without and with Zn^{2+}, respectively (frequency 10 mHz and 3% amplitude). Average values ± SEM are result of three independent experiments. The semi-difference between $\varepsilon + G$ and $\varepsilon - G$ corresponds to the shear viscoelasticity.

response was smaller in all cases than the shear elastic response, but it was larger for insulin with Zn^{2+}. However, Krägel et al. [41] suggested that a direct method, such as barrier oscillations, may lead to misinterpretation of the viscous data (ε'' and G'') because such a technique does not account for viscous interactions between the interface and the bulk. As a consequence, our viscous values for dilatational and shear moduli may possibly be underestimated, and indirect methods such as channel surface viscometer, deep-channel surface viscometer or the rotating knife-edge wall surface viscometer may be necessary to precisely ascertain changes of viscosity in the films.

Both insulin monolayers without Zn^{2+} developed only a dilatational modulus. This is consistent with the behavior of some polymer monolayers where the real and imaginary components of the dynamic shear modulus are negligible, at least at low frequencies [42,43]. However, both insulins with Zn^{2+}exhibited well-defined shear moduli, much larger in the case of Reg-insulin. The shear modulus describes the system's response to changes in shape that occur at constant area [19]. It is important to note that when a protein monolayer shows a shear modulus it means that it becomes a nominal solid as a result of developing a contact network [19]. In colloidal systems, a shear modulus can develop from one of two processes: either by the formation of bonds between different particles, driven by attractive interactions and leading to intertwined or branched percolating structures, or by dynamical arrest due to crowding, where each particle is effectively caged by neighboring hard-core repulsive interactions [19,44]. Therefore, the presence of Zn^{2+} in the subphase appears to act as an organizing factor for Reg-insulin and Asp-insulin monolayers, but mainly for Reg-insulin by which it is able to generate a viscoelastic network (beyond hexamer formation) that may account for the shear modulus observed.

For some proteins such as bovine serum albumin, a monotonic increase of ε modulus to high values with increasing adsorption or Π, a behavior ascribed to globular non-flexible proteins [45], is observed. This type of increase was observed in Reg-insulin with Zn^{2+} up to Π = 5 mN/m, with an $\varepsilon \approx 100$ mN/m (Fig. 8A and B). This behavior clearly differentiates from the viscoelasticity of Reg-insulin without Zn^{2+} and from Asp-insulin (irrespective of Zn^{2+}presence). In these films we observed a smaller increase of the maximum value of ε (between half and one third) followed by a plateau or even a small decrease, similar to that found for flexible proteins like β-casein [45].

Finally, in relation to our previous work on the hippocampal pyramidal neuron polarization differentially induced by the surface organization of Reg-insulin [11], these results suggest a possible correlation of the neuronal response to the different viscoelastic behavior observed for Reg-insulin as a function of Π. In that work, we observed that the neural cells grew and polarized less favorably when the insulin surface was more closely packed [11]. Interestingly, correlation of these results with the surface rheology described indicates that neurons can polarize when the insulin film used as a substrate for growth exhibits a relatively small dilatational and shear elasticity. This suggests that a more fluid, less elastic,

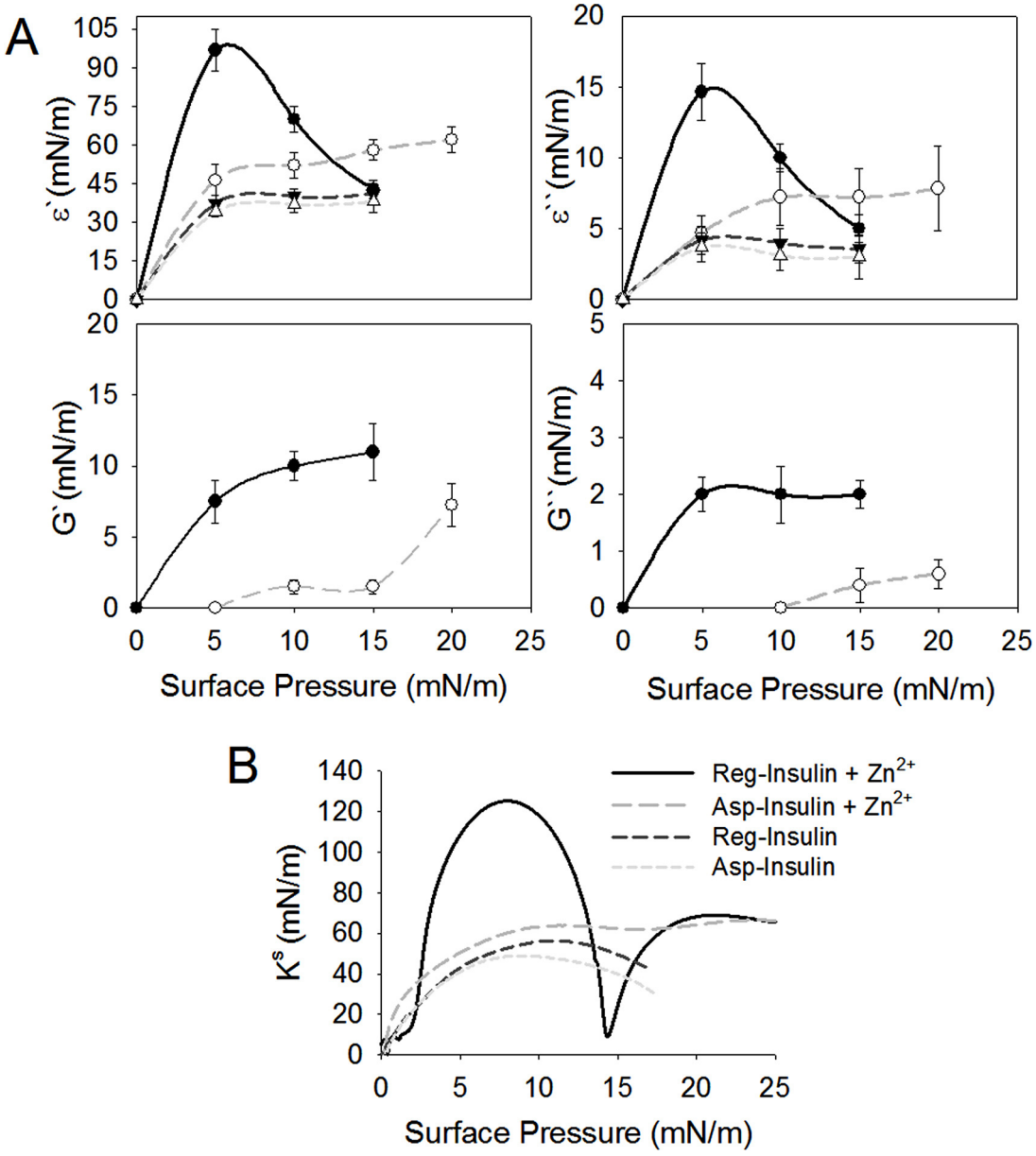

Fig. 8. Dilatational and shear moduli. (A) Reg-insulin with Zn^{2+} (●); Asp-insulin with Zn^{2+} (○); Reg-insulin (▼) and Asp-insulin (△) without Zn^{2+}. ε' and ε'' are the elastic and viscous response of the dilatational moduli, respectively. G' and G'' are the elastic and viscous response of the shear moduli, respectively. Compression speed: 0.5 mm/min. Average values ± SEM are result of three independent experiments. (B) Surface compressional moduli, K^s, in the range of 0–30 mN/m (compression speed: 0.5 mm/min).

surface provided by a Le viscoelastic network of insulin may constitute a more suitable substrate on which the cell membrane can become adapted, in order to optimize neuron recognition, adherence and triggering of specific polarization responses following IGF-1 receptor activation [11].

6. Conclusions

The interfacial behavior of Reg-insulin and Asp-insulin was critically affected by Zn^{2+} in the subphase. This cation induced a condensed-like behavior in the compression isotherms, marked variations of the surface electrostatics and considerable hysteresis of the monolayer organization of both insulins. With Zn^{2+}, a dilatational response to the surface perturbation was observed in both types of insulin, with defined shear moduli that was higher for Reg-insulin. The development of the latter indicates a behavior resembling a nominal solid, more apparent for Reg-insulin than for Asp-insulin, suggesting the presence of viscoelastic networks at the surface.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2013.11.031.

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