Influence of ionic strength and beta2-glycoprotein I concentration on agglutination of like-charged phospholipid membranes

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ABSTRACT

The effect of ionic strength on adhesion between negatively charged giant unilamellar vesicles induced by beta2-glycoprotein I (β2-GPI) was studied experimentally and theoretically. Measuring the effective angle of contact between adhering vesicles indicated that the strength of adhesion between vesicles decreases with increasing ionic strength, and increases with concentration of β2-GPI. In the theoretical part we focused on the study of the average orientation of β2-GPI near the charged membrane and its role in mediating the attractive interactions between the vesicles. β2-GPI proteins were modelled as rods with internal distribution of electric charge. The predictions of Monte Carlo simulations show orthogonal orientation of some of the membrane attached β2-GPI in narrow gap between two vesicles. On the contrary, at larger distances between vesicles the proteins are parallelly attached to the membrane surface. A local minimum of the free energy corresponding to β2-GPI-mediated adhesion of two neighbouring vesicles was predicted. The strength of adhesion was confirmed to decrease at high ionic strength.

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1. Introduction

Beta2-glycoprotein I (β2-GPI) is a highly abundant 50 kDa phospholipid-binding plasma glycoprotein and a principal autoantigen for antiphospholipid antibodies in the antiphospholipid syndrome (APS) – a disease characterized by vascular thrombosis and fetal loss [1]. In patients with APS antiphospholipid antibodies recognize and bind to β2-GPI when it is bound to anionic surfaces, such as negatively charged phospholipid membranes. The interaction between β2-GPI and negatively charged phospholipids is important also for the other proposed functions of β2-GPI, e.g. for clearance of apoptotic cells [2] and negatively charged liposomes [3,4].

The molecule of β2-GPI consists of four short consensus repeat (SCR) domains (domains I–IV) and a C-terminal fifth domain (domain V), which is constructed on a SCR-like core but has an additional hydrophobic six residue insertion and a C-terminal extension of 19 amino acids. Although both terminal domains – domains I and V of β2-GPI can interact with anionic phospholipids [5], domain V is the principal phospholipid-binding domain [6]. Specifically, the highly positively charged lysine-rich C-terminal extension of domain V interacts electrostatically with anionic phospholipids and the hydrophobic loop of domain V putatively inserts into the lipid bilayer [7–13].

Recently it has been shown that β2-GPI can exist in at least two different conformations [14]. The circulating β2-GPI in plasma exhibits a closed, circular conformation that is maintained by the interaction between domains I and V. In the presence of anionic phospholipids or under high salt and high pH conditions the circular conformation is reversibly converted into an open elongated conformation, in which domain V can interact with anionic phospholipids [14,15]. The elongated conformation of β2-GPI is comparable to a fish hook J-shaped crystal structure of β2-GPI molecule with overall dimensions of (13.0–13.2) nm × (7.2–8.5) nm × 2.0 nm [7,8].

In contrast to domain V the interaction of domain I with negatively charged phospholipids occurs only in the solutions with low ionic strength and subsequently to the primary phospholipid binding of domain V [16] where the domain I can interact with the same membrane as the domain V or with the second adjacent membrane. This is in line with the observations that under low ionic strength conditions the interaction of β2-GPI with target membranes, such
as negatively charged small and large unilamellar vesicles [17–21] and mitochondria [22] leads to their adhesion or agglutination and subsequent precipitation. The agglutination of small and large unilamellar vesicles does not occur under higher ionic strength conditions (150 mmol/l) [21], when domain I is presumably detached from the membrane.

Membrane binding of both domains V and I may be therefore important for β2-GPI-induced membrane agglutination [18,20,21], however the exact mechanism driving β2-GPI-induced membrane agglutination is still unknown [21]. It was suggested that agglutination and subsequent precipitation of negatively charged small unilamellar vesicles in the presence of β2-GPI could be caused by the increased hydrophobicity of the target vesicle membrane after multivalent binding of β2-GPI with target membrane [5,21]. Specifically, charge–charge interactions between β2-GPI and anionic phospholipids could shield the charge repulsions that may impede protein–protein and anionic amphiphile–anionic amphiphile interactions. This could result in decreased solubility of membranes in aqueous media, thus providing a driving force for the formation of large vesicle aggregates [21]. Alternatively, β2-GPI was suggested to cross-link/cross-bridge adjacent negatively charged vesicle membranes through positive charges within domains V and I, thus inducing their adhesion/agglutination [18,19,23]. This cross-bridging mechanism was explained theoretically as a consequence of an orientational and positional ordering of polyionic β2-GPI molecules within the gradient of a local electric field between two negatively charged membrane surfaces. It was suggested that such system attains its free energy minimum at a distance between the surfaces equal to the length of the polyionic molecule, yielding a cross-bridging configuration of β2-GPI [24].

The aim of the present work was to study further, experimentally and theoretically, β2-GPI-mediated agglutination/adhesion between negatively charged planar phospholipid membranes, focusing on the influence of β2-GPI concentration and ionic strength on β2-GPI-mediated membrane adhesion. Adsorption of various charged proteins to charged biological surfaces was experimentally observed which can be explained in terms of electrostatic forces between the charged surface and charged proteins [25,26]. In the theoretical part of the work the orientation of polyionic β2-GPI in the space between negatively charged membranes at different distances between the membranes was studied using Monte Carlo (MC) simulations of electrostatic forces in the system. The results of MC simulations are utilized in interpretation of experimental results. The effect of ionic strength on β2-GPI-mediated vesicle adhesion is considered separately within the mean field Poisson–Boltzmann (PB) approach.

2. Materials and methods

2.1. Preparation of giant phospholipid vesicles

Lipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), 1,1',2,2'-tetraeyloley-cardiolipin (CL) and plant cholesterol (Avanti Polar Lipids, Inc., Alabaster, AL) were dissolved in chloroform or chloroform/methanol mixture at the concentration of 1 mg/ml. To prepare POPS/CL-giant phospholipid vesicles (GPVs) POPC, cholesterol and POPS/CL were combined in the proportion of 7:1:2 (v/v/v) and for neutral GPVs POPC and cholesterol were combined in the proportion of 4:1 (v/v). GPVs were prepared by the modified electroformation method, originally proposed by Angelova et al. [27]. After the electroformation, the solution containing 0.192 mol/l glucose and 0.28 mol/l PBS (v/v = 9:1) was added to the sucrose GPV suspension (v/v = 3:5) and the vesicles were left to sediment under gravity in a low vacuum at room temperature for one day.

2.2. β2-glycoprotein I

β2-GPI was isolated from pooled human plasma by perchloric acid precipitation as described in [28] and was dissolved in 0.28 mol/l PBS, pH 7.4. The concentration of β2-GPI isolated from human plasma was determined using Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). In some experiments commercially available purified human β2-GPI (Hyphen Biomed, Neuville-sur-Oise, France), dissolved in 0.28 mol/l PBS pH 7.4, was used. The final concentrations of β2-GPI were 10–200 μg/ml in β2-GPI concentration experiments and 180 μg/ml in ionic strength experiments. With regard to the data published in [14] and the method we used to isolate β2-GPI from human plasma, human plasma-isolated β2-GPI was hypothesized to exist predominantly in the closed circular conformation. Furthermore, in accordance with data published in [14,15] we expected that in the presence of negatively charged GPVs β2-GPI will exist predominantly in the elongated conformation.

2.3. Experimental procedure – GPV characterization and observation

The experiments were performed in a home-made observation chamber as described in Ambrožić et al. [17] at 22 °C or 37 °C and pH 7.4. For ionic strength experiments POPS-GPV suspensions of different ionic strengths were prepared by combining different volumes of GPV-sugar-PBS suspension with different volumes of 150 mmol/l NaCl solution. The final ionic strength in the experiments ranged from 22 mmol/l to 144 mmol/l. β2-GPI was added to the sugar-PBS-NaCl suspension of GPVs in the observation chamber. PBS containing no β2-GPI was used to control for potential spontaneous adhesion between GPVs.

The experiments were observed by an inverted microscope Zeiss Axiovert 200 (Carl Zeiss MicroImaging, Jena, Germany) with phase contrast optics and were recorded by the Sony XC77-CE (Sony Corporation, Tokyo, Japan) or VisiCam 1280 Cameras (Visitron Systems, Puchheim, Germany). The images of adhered GPVs were acquired using MatLab (The MathWorks Inc., Natick, MA) or MetaMorph Imaging System (Visitron Systems, Puchheim, Germany) softwares. Under the phase contrast microscope GPVs containing sucrose solution appeared darker in comparison to the surrounding sugar-PBS-NaCl solution due to the differences in refraction indexes of the solutions.

2.4. Measurement of adhesion between GPVs

The strength of adhesion between GPVs was determined using the semiquantitative method of an effective angle of contact [29,30]. The effective angles of contact between adhering GPVs were measured in two-dimensional images acquired 20–30 min after the addition of β2-GPI into the GPV suspension, using Image J software (National Instruments of Health, Bethesda, MD). On average, 200 effective angles of contact were measured in each experiment, and the average effective angle of contact φw was calculated. The larger average effective angle of contact represents stronger adhesion between GPVs, while the smaller average effective angle of contact represents weaker adhesion between GPVs (Fig. 1).

Statistical analysis was performed using SPSS 15.0 software (SPSS Inc., Chicago, IL). For the average effective angle of contact between adhering GPVs descriptive statistical parameters (average, standard deviation, frequencies, frequency distribution) were calculated.
3. Experimental results

3.1. Influence of β2-glycoprotein I concentration on adhesion of cardiolipin-GPV

The influence of increasing β2-GPI concentration on the adhesion of CL-GPVs was studied under low ionic strength conditions (30 mmol/l). The adhesion of CL-GPVs increased with increasing concentration of β2-GPI (Fig. 2). A rather steep increase in the average effective angle of contact between GPVs (from 25° to 80°) was observed as β2-GPI concentration was raised from 10 to 50 μg/ml. Maximal adhesion with average effective angle of contact of approximately ϕe = 100° was reached at β2-GPI concentration larger than 150 μg/ml, which is within the physiological range of β2-GPI concentrations in human plasma (150–300 μg/ml). The similar concentration-dependent β2-GPI-mediated adhesion was observed also for POPS-GPVs, as described in reference [19]. There was no adhesion between neutral, GPVs, containing POPC and cholesterol (data not shown).

3.2. Influence of ionic strength on β2-glycoprotein I-induced adhesion of GPVs

β2-GPI-induced adhesion of negatively charged POPS GPVs, containing physiologically relevant levels of phosphatidylserine (10–20 mass%), was ionic strength dependent. Increasing the ionic strength of POPS-GPV suspension from 49 to 126 mmol/l and from 22 to 94 mmol/l resulted in almost a linear decrease of the average effective angle of contact from 103 ± 16° to 39 ± 10° and from 112 ± 25° to 43 ± 10°, respectively (Fig. 3).

4. Theory

4.1. Monte Carlo simulations of β2-GPI binding and orientation to the membrane surface

In our theoretical model, the two interacting membranes of GPVs were described as two negatively charged planar surfaces immersed in an ionic aqueous solution containing positively charged proteins (Fig. 4). We used MC simulations of the Coulomb interactions in the system to determine the average distribution and orientation of proteins between the charged membrane surface in the dielectric medium (water with relative permittivity εr = 80). For the sake of simplicity, we modelled a single β2-GPI molecule as an infinitely thin rod with a length of L = 10 nm and two charges with valency Z at both tips of the rod, see Fig. 4.
We did not take into account stearic restrictions due to attached proteins. The charge distribution within a single β2-GPI molecule was taken into account by two positively charged regions on the domains I and V. A charge of valency \( Z = 6 \) was ascribed to both of them. Learning from previous biochemical studies on the β2-GPI structure \([11, 31, 7, 8, 32]\), we expected our model of β2-GPI to be appropriate to study the orientation and distribution of β2-GPI proteins between two charged vesicles (Fig. 4) in planar geometry.

The simulation box consists of two planar charged surfaces with area \( Y^2 \) and surface charged density \( \sigma_m \), which are placed with an inter-surface distance \( D \). Within the box a fixed number of rod-like particles representing β2-GPI is included. In the model we assumed that electrostatic field changes only along the \( x \) axis. The electroneutrality of the system is fulfilled. In each MC step, the randomly chosen particle is translated or rotated. The selection of the type of move (rotational or translational) has the same probability 0.5. The electrostatic potential is computed by combination of methods of Lekner and Sperb \([33, 34]\) and is evaluated by the Metropolis algorithm \([35]\) in order to find a minimum of electrostatic potential.

The MC simulations provided us with average volume distribution of protein charges and a protein average orientation between the two charged membrane surfaces. Proteins are considered to be orthogonally oriented as long as the angle between the longitudinal axis of the protein and \( x \)-axis deviates within angle \( \theta = \pm \pi/16 \).

The series of figures in Fig. 5 show the calculated volume charge distribution between the two charged surfaces for different inter-membrane distances \( D \). When the two charged surfaces are approximately at a distance equal to the length of the protein \( L \), the volume charge distribution shows peaks close to both surfaces. These peaks represent the charge of orthogonally oriented proteins, which is essential in explaining the experimentally observed adhesion. The similarity of the volume charge distribution given in Fig. 5A and F, where the peaks are found only at the charged surfaces, may lead to wrong conclusions since these two cases are very different. Namely the values of average order parameter \( S = \langle 3 \cos^2(\theta) - 1/2 \rangle \) are \( S_A = 0.5427 \) and \( S_P = -0.4697 \), which indicates a moderate orthogonal orientation in the first case (Fig. 5A) and a completely parallel orientation for the second case (Fig. 5F).

In order to show how the protein average orientation is affected by the inter-surface distance and by the electrostatic field strength, we plotted the percentage of the orthogonally oriented proteins in Fig. 6. The figure shows the results for two different surface charge densities. More proteins are orthogonally oriented when the membrane surfaces are closer together. As their distance increases more

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**Fig. 5.** Electric volume charge distribution of proteins between two negatively charged membranes are shown for six different inter-membrane distances \( D = \{10.0, 12.5, 14.0, 16.0, 17.5, 20.0\} \) nm, \( \sigma_m = -0.07\) As/m², \( Z = 6 \), \( L = 10 \) nm. Proteins which seem to mediate attraction between the negatively charged membranes, are shown schematically. In panels A–E some proteins are oriented orthogonally to the membrane surface, in panel F almost all proteins prefer parallel orientation and cannot mediate attraction.
proteins are oriented parallelly to the surfaces. This effect is more pronounced for higher surface charge density of the membrane.

4.2. Evaluation of the free energy within Poisson–Boltzmann model

In this section, evaluation of the free energy of the system for different values of the ionic strength is described within the mean-field Poisson–Boltzmann model. Based on the MC results presented in Fig. 5, we assume that most of the β2-GPI proteins accumulate close to the membrane surface and some of them are orientated orthogonally to the surface (Fig. 7). Their orientation is dependent on the inter-surface distance $D$. Accordingly, the positively charged tips of bound and oriented proteins can be treated as a new charged layer at the distance $L$ from the membrane surface with surface charge density $\sigma_p$ (Fig. 7). The positive charges of the second tips and the tips of proteins which are bound parallel to the membrane surface would reduce the membrane surface charge density to the value of $(\sigma_m + \sigma_{pp})$; note that $\sigma_m$ is negative. Such a system can be described by four boundless planar, parallel, charged surfaces immersed in a solution composed of monovalent salt ions with bulk number density $n_s$, see Fig. 7. As the distribution of the orthogonally oriented proteins changes with inter-membrane distance, values of $\sigma_p$ and $\sigma_{pp}$ also vary with $D$. Note that according to our assumptions and results of our MC model we kept $(\sigma_{nm}(D) + \sigma_{pp}(D) + \sigma_p(D)) = 0$ in order to maintain consistency with the results of the MC simulation. This assumption means that the total surface charge is neutralized by the charges of the proteins, see also Fig. 5. The dependence of the number of orthogonally oriented proteins on distance $D$ is approximated by linear functions based on the results of our MC simulation $f_i(D)$, where $i = 1, 2, 3, 4$; see Fig. 6.

In order to determine electrostatic potential ($\phi$) in the system, we solved Poisson–Boltzmann equation for 1:1 electrolyte solution [36]:

$$\frac{d^2 \Psi(x)}{dx^2} = \kappa^2 \sinh(\Psi(x)),$$

(1)

where we introduced the reduced electrostatic potential $\Psi = \epsilon_0 \phi / kT$ and $\kappa$ is inverse value of the Debye length $\lambda_D = k^{-1} = \sqrt{\epsilon_0 \epsilon_r kT / 2n_s e^2}$ [36]. Here $\epsilon_0$ is the elementary charge, $kT$ is the thermal energy, $\epsilon_r$ is the vacuum permittivity, $\epsilon_r$ is the relative permittivity of water and $n_s$ is the number density of the monovalent salt in the bulk solution corresponding to the values from the experimental part of the paper. Six boundary conditions for Eq. (1) are in accordance with condition of electroneutrality and are given in Appendix B.

The free energy of the system per unit area is characterized by the energy of the electrostatic field (first term) and entropic contribution of salt ions (second term) [see for example [37]]:

$$F/A = \int_0^D \left( \frac{1}{2} \epsilon_r \epsilon_0 \left( \frac{d \Psi}{dx} \right)^2 + kT \sum_{j=-,}^{+} \left(n_j \ln \left( \frac{n_j}{n_s} \right) - (n_j - n_s) \right) \right) dx,$$

(2)

where $n_j$ are the number densities of anions ($j = -$) and cations ($j = +$) in the salt solution. The contribution of bound proteins to the free energy is included through boundary conditions.

The free energy of the system as a function of inter-membrane distance $D$ for different ionic strengths $n_j/N_A$ and $\sigma_m = -0.070$As/m$^2$ is depicted in Fig. 8. The existence of an energetic barrier (around 0.03 kT per nm$^2$ which is 0.0165 kT per molecule)
at $D \equiv 8/5L$ shows that the attraction between the two surfaces can take place only after crossing over the energy barrier. The driving force for crossing the barrier can be for example the kinetic energy of the vesicles freely moving in the suspension. After crossing the barrier, the vesicles are entrapped in energy minimum, which keeps them in the agglutinated state. The strength of adhesion is stronger when the depth of energy minimum is higher (see Fig. 8). The energy barrier and the depth of energy minimum decrease with increasing ionic strength.

4.3. Comparison between theory and experiments

Fig. 9 shows the scaled depth of the free energy minimum $\Delta F/\Delta F_0$ as a function of the salt number density $n_s$. For comparison, the normalized average effective angle of contact $\psi_c/\psi_{c,0}$, where values of $\psi_c$ are taken from the experimental results presented in Fig. 3, is also plotted in the same figure. Both, the experimental as well as theoretical results on GPVs in the presence of $\beta_2$-GPI show a decrease of adhesion between GPVs with increasing salt concentration. The experimentally determined $\psi_c/\psi_{c,0}$ decreases almost linearly with salt concentration. The normalized free energy barrier as well as the normalized average effective angle of contact decrease more strongly for higher surface charge density of the membrane.

5. Discussion and conclusions

We showed theoretically that positively charged rod-like particles (in our case representing $\beta_2$-GPI molecules) can be strongly electrostatically attracted and oriented to the charged surface at higher surface charge densities. The attraction is the result of the spatial distribution of charges within the multivalent particles, i.e. the result of intra-ionic correlations. These intra-ionic correlations are described in the model via the fixed distance between the electric charges within the single rod-like particle and contribute to the attractive force between the equally charged membrane surfaces. Analysis of the average orientation of rod-like particles suggests that the bridging mechanism leads to a local minimum in the free energy of the system, corresponding to the equilibrium distance between the like-charged surfaces. The equilibrium distance between the charged surfaces is approximately equal to the length of the rod-like particles (Fig. 10). We would like to point out that the interaction between membrane and $\beta_2$-GPI in our model is only of electrostatic nature and that the attraction between vesicles is possible also without taking into account the possible role of the hydrophobic loop of $\beta_2$-GPI.

The effect of ionic strength on the strength of adhesion between like-charged membrane surfaces is based on screening of the potential [38] where the strength of adhesion is smaller for higher ionic strength. Screening affects not only the membrane surface, but also the positively charged surfaces formed from charged tips of the oriented proteins (Fig. 7). The screening effect, therefore, makes the interaction between like-charged surfaces weaker.

We are aware of the simplification in our MC model where the effect of ionic strength was omitted in the first step. Nevertheless, we consider our approach adequate to discuss possible mechanism of the origin of attractive forces between like-charged membrane surfaces mediated by oppositely charged rod-like particles. Due to the above mentioned assumption, the predictions of the MC simulations for higher values of ionic strength may be less realistic. There would probably be fewer orthogonally oriented proteins because of the electric double layer formed from the salt ions and because of the interaction between salt ions and charged proteins. Consequently, the predicted energy minimum and energy barrier would be slightly lower and thus closer to the experimental results. Our results of MC simulations were compared to the results of the complex study of Kim et al. [39] in the PB regime and a good agreement was found.

We have presented an insight into the mechanism of average orientation of rod-like proteins near charged membrane surfaces. It is shown that intra-ionic correlations can affect the electrostatic properties and induce attractive interaction between like-charged surfaces. By exploring the average orientation of the charged rod-like particles, we showed in our MC simulation that even when the macroscopic volume charge distribution of suspension of...
charged rod-like particles is similar, the orientation of the charged rod-like particles near the charged surface or between the charged surfaces can be significantly different (compare Fig. 5A and F).

We would like to point out to the difference in orientational ordering between thin rod-like particles and spheroïdal particles where the hard-core interactions between the spheroïdal particle and the charged surfaces were taken into account by means of the distance of the closest approach (i.e. spheroïdal charged particles are treated like hard spheres when interacting with the charged surface) [40]. In the latter case, all spheroïdal particles are on average oriented orthogonally to the charged surface even for larger inter-membrane distances \((D > 2L)\), if the surface charge density is high enough. Therefore the adhesion between like-charged surfaces mediated by spheroïdal particles is stronger and acts at longer distances as in the case of rod-like particles. In general, proteins are neither infinitely thin rods nor perfect spheres but geometric shape of most proteins lies somewhere between the two above mentioned extreme shapes.

To conclude, in the present paper we have elucidated experimentally and theoretically the effect of ionic strength on \(\beta_2\)-GPI-mediated adhesion between negatively charged unilamellar vesicles. In the experiments we found that higher content of \(\beta_2\)-GPI in the solution causes stronger adhesion, while the increasing ionic strength decreases the strength of adhesion for given concentration of \(\beta_2\)-GPI. Our theoretical model showed that the average orientation of rod-like proteins with internal charge distribution changes with the membrane separation \(D\) leading to the creation of an energy barrier between the two vesicle surfaces. After crossing this barrier, the vesicles stick together since they are entrapped in a local minimum of the free energy of the system. The energy minimum and the energy barrier was found to be lower for higher ionic strength media.

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Appendix A. Surface charge density of POPS-containing GPsVs

The surface charge density of POPS-containing GPsVs is assessed as follows:

\[
\sigma(GPV) = \frac{(\text{n}_{\text{POPS}} \cdot \text{Z}_{\text{POPS}} + \text{n}_{\text{POPC}} \cdot \text{Z}_{\text{POPC}}) \varepsilon_0}{(\text{n}_{\text{POPS}} + \text{n}_{\text{POPC}}) \varepsilon_0},
\]

where \(\text{n}_{\text{POPS}}\) and \(\text{n}_{\text{POPC}}\) are moles of POPS and POPC, respectively, in lipid mixture used for electroformation of GPsVs; \(\text{Z}_{\text{POPS}} = -1\) and \(\text{Z}_{\text{POPC}} = 0\) are the net charge valencies of POPS and POPC polar heads, respectively; and \(\alpha = 0.55\) nm\(^2\) \([41]\) is the average surface area occupied by one molecule of POPS or POPC in the lateral plane of membrane. It was assumed that the molar ratio of POPS and POPC molecules in GP membrane corresponds to POPS/POPC molar ratio in lipid mixture used for electroformation.

Appendix B. Boundary conditions for four-layer Poisson–Boltzmann equation

Six boundary conditions at four surfaces (Fig. 7), which ensure the continuous and smooth reduced electrostatic field and reduced electrostatic potential, are:

\[
\frac{d\Psi}{dx} (x = 0) = 0,
\]

\[
\Psi(x = L_1) = \Psi(x = L_2).
\]

References


