

VACCINE FORMULATION: ADSORPTION OF *Plasmodium falciparum* MSP-1 PEPTIDE 1585 ON ALUMINIUM HYDROXIDE

FORMULACIÓN DE VACUNAS: ADSORCIÓN SOBRE HIDRÓXIDO DE ALUMINIO DEL PEPTIDO 1585 DERIVADO DE LA PROTEINA MSP-1 DE *Plasmodium falciparum*

Mary TRUJILLO¹*, Luz M. SALAZAR¹, Jesús VALENCIA¹

Received: 17 January 2011 Accepted: 25 May 2011

ABSTRACT

The *Plasmodium falciparum* merozoite surface protein 1 has been studied due to its potential to become a vaccine; likewise, the peptide 1585 which is located in the 42-kDa amino-terminal fragment induces protective immunity in primates. Despite the importance of antigen adsorption in the formulation and production of vaccines containing aluminium adjuvant, the protein fragment adsorption on aluminium hydroxide has not been thoroughly studied. Electrostatic attraction, hydrophobic interaction and ligand exchange have been identified as the major mechanisms involved in antigen retention on the adsorbent surface. Peptide 1585 was synthesized, and its solubility, adsorption on aluminium hydroxide, as well as its molecule release have been studied here. Results allowed us to raise a model for the adsorption and release of this peptide, which are important parameters to establish optimal conditions for peptide-adsorbent interaction and, therefore, their response as a vaccine. Results also established the reversibility of such process due to the phosphate ion effect.

Thus, this work provides a starting point for research works, leading to further development of vaccine formulations containing highly purified synthetic antigens adsorbed on aluminium adjuvant.

Keywords: Adsorption, aluminium hydroxide, peptide, vaccine, *Plasmodium falciparum*.

RESUMEN

La proteína de superficie del merozoito de *Plasmodium falciparum*, MSP-1, es reconocida como candidata a vacuna; asimismo, el péptido 1585, situado en la región amino terminal de 42- KDa, induce inmunidad protectora en primates. A pesar de la importancia de la adsorción del antígeno en la formulación y producción de vacunas que contienen hidróxido de aluminio como adyuvante, la adsorción del fragmento proteico no ha sido estudiada. Los principales mecanismos que se han identificado como responsables de la retención de un antígeno sobre la superficie adsorbente son las interacciones electrostáticas, las interacciones hidrofóbicas y el intercambio de ligando.

En este trabajo se sintetizó el péptido 1585 para estudiar la solubilidad, la adsorción sobre hidróxido de aluminio y la liberación de la molécula. Los resultados permitieron plantear el modelo para la adsorción y la liberación de este péptido, parámetros importantes para establecer las condiciones óptimas para la interacción péptido- adsorbente y por ende, su respuesta como vacuna. Los resultados también mostraron la reversibilidad del proceso debido al efecto del ión fosfato.

Palabras clave: adsorción, hidróxido de aluminio, péptido, vacuna, *Plasmodium falciparum*.

¹ Facultad de Ciencias, Universidad Nacional de Colombia, sede Bogotá. Carrera 30 No. 45-03, Tel.: +57-1-3165000 ext. 14620 Fax: +57-1-3165000 ext. 14640. Bogotá, Colombia.

* Corresponding author: mtrujillo@unal.edu.co

INTRODUCTION

Most synthetic or recombinant vaccines have shown to be safe; however, their reduced immunogenicity entails the use of immune adjuvants capable of amplifying and directing the host immune response against the antigen.

Aluminium hydroxide (AH) and aluminium phosphate (AP) containing adjuvants are known as deposition adjuvants, since they adsorb the antigen to increase their biological and immunological half-life, and they can be detected by the immune system due to their gradual release.

Studying the parameters that govern peptide adsorption and release onto AH will lead to establishing the necessary conditions for achieving the best adsorbate-adsorbent interaction and, thus, optimizing such process and the vaccine formulation.

The mechanisms underlying peptide adsorption in vaccines formulated with AH are studied by plotting adsorption isotherms at different solution concentrations, traditionally determined by decreasing the quantity of antigen present in the solution.

Protein adsorption studies on AH (1-3) have shown that these molecules are retained according to Langmuir's adsorption model (4), which poses that all adsorption sites are energetically equivalent, that no intermolecular interaction occurs in the system, and that adsorption is accompanied by monolayer formation.

Langmuir's equation has been used as a semi-quantitative approach for characterising physicochemical adsorption parameters, such as adsorption capacity and adsorption coefficient (Kd). These parameters have been successfully applied to predict the competitive effect with other proteins, which should be taken into account at the moment of manufacturing multi-component vaccines adsorbed on AH (5-7).

Other studies have shown that intra and intermolecular interactions may occur depending on the protein structure, causing the formation of multiple antigen layers on the adsorbent surface, which is a situation further favoured at high protein concentrations (2, 5-9).

Models for interpreting the characteristics of adsorption isotherms from solutions describe monolayer or bilayer formation; however, such scheme differs from recent proposals suggesting molecule aggregation on the adsorbent surface.

Protein adsorption on solids is usually irreversible and there is no significant loss of retained protein; however, this condition may be altered when the surface remains immersed in dissolution media.

When the dissolution does not result in protein desorption, proteins may be released from the surface by adding other molecules that can be retained on the surface through an exchange mechanism, in which the adsorption of higher affinity molecules favours polypeptide replacement. Such adsorbed molecule exchange mechanism can be heteromolecular (i.e. proteins displaced by another protein) or homomolecular (i.e. in systems where adsorbed and dissolved molecules are dynamically exchanged between adsorbed and dissolved states) (10).

Depending on the system, the exchange may take minutes or even days and the protein being released does not necessarily keep the same structural characteristics it exhibited before being adsorbed. Adsorption may thus be reversible or irreversible, depending on the protein structure before and after being adsorbed (11).

Antigens adsorbed on aluminium containing adjuvants are exposed to two different environments: the vaccine's components before they are administered, and the interstitial fluid following intramuscular or subcutaneous administration. Many substances contained in the interstitial fluid, such as phosphate (12), citrate ions (13, 14) and interstitial proteins (15) can rapidly alter the degree of antigen adsorption on AH.

Peptide 1585 (¹E²V³L⁴Y⁵L⁶K⁷P⁸L⁹A¹⁰G¹¹V¹²Y¹³R¹⁴S¹⁵L¹⁶K¹⁷K¹⁸Q¹⁹L²⁰E) adsorption was studied in this work; it is a mainstream malaria vaccine candidate derived from *Plasmodium falciparum* MSP1. It was found that peptide adsorption on AH generated complex isotherms, suggesting the formation of several layers on the adsorbent. A model of the structure of the adsorbed layer is proposed, constituting one of the first kinetic experimental results reported, which are aimed to understanding the molecular interactions between the peptide vaccine candidate and the adsorbent.

MATERIALS AND METHODS

Peptide 1585 synthesis and characterisation

Peptide 1585 was obtained by the solid-phase multiple peptide synthesis method proposed by

Merrifield, 1963 (16) and improved by Houghten, 1985 (17). Crude peptide was purified by RP-HPLC. Peptide purity was verified on an analytical Lichrosorb® C18 column using 0.05% TFA in water (solvent A), 0.05% TFA in ACN (solvent B), and a 0 - 70% gradient of solvent B for 30 min. Peptide molecular mass was determined on a Bruker MALDI-TOF mass spectrometer. The peptide 1585 secondary structure was assessed by circular dichroism (CD) spectroscopy using a JASCO® spectropolarimeter calibrated with d-10-camphorsulphonic acid; and a 0.1 mM solution of purified peptide was used in 30% TFE-H₂O at 298 K.

Adsorption isotherms on AH

0.5 to 12 mg/mL (0.2 - 5 mmol/L) concentration peptide solutions were prepared at constant temperature (273 K) in 0.9% sodium chloride with a 7 ± 0.1 pH. An AH equivalent to 1.6 mg of Al/mL (18) was added, shaking the mixture for 12 hours at 150 rpm. Peptide concentration, before and after adsorption was determined by spectrophotometry at 570 nm using bicinchoninic acid (BCA) (19-21). The amount adsorbed in mmol/mg Al was determined by the difference between these values, and it was plotted in terms of the initial solution concentration.

Release isotherms

Peptide 1585 adsorbed on AH at a 1 mmol/L concentration in aqueous solution was stored for 24 hours at 277 K; a 100 mM sodium phosphate solution (pH 7.0) was then added to obtain a final phosphate ion concentration of 4 mM (12).

The mixture was stirred at 310 K and supernatant placed in aliquots, replacing the solvent to obtain a constant volume. The samples were spun at 8,000 rpm for 5 min and the amount of peptide in the solution was determined, using the standard micro-BCA protocol (Pierce) (19 - 21) and the purified peptide pattern curve.

Molecular docking

Docking was performed with a molecular calculation package (2000, Accelrys, San Diego, CA) on an Indigo II work station (Silicon Graphics), using the previously obtained NMR structural model (22), and calculating Van Der Waals energy (VDWE), electrostatic or Coulomb energy (CE), and the combination of both or total energy (TE).

RESULTS AND DISCUSSION

Peptide characterisation

The chromatographic analysis of peptide 1585 in pure state, gave as result a retention time of 23.6 minutes, and mass spectrum showed a 2,348.8 Dalton signal, which corresponds to the expected peptide molecular mass (data no shown). The secondary structure elements forming peptide 1585 tridimensional structure are shown in the DC spectrum in figure 1. The curve (a) (solid line) has a maximum molar ellipticity at 193 nm (transition π - π^* positive with Θ in 30,000), and a minimum molar ellipticity at 208 nm (transition π - π^* negative and 222 nm transition n- π^*), which is characteristic of a helicoidal structure (23).

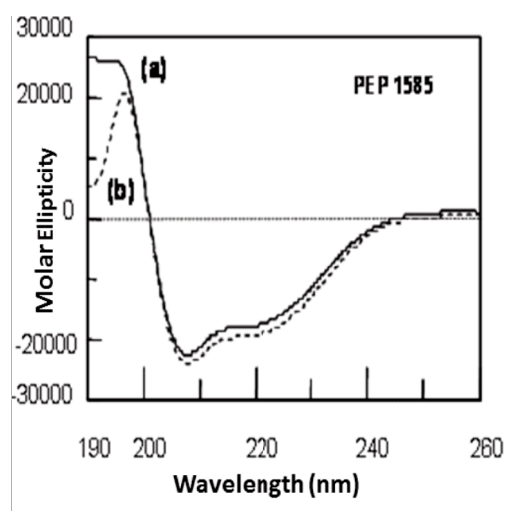


Figure 1. Peptide 1585 CD spectrum. The curve shape in the spectrum shows a typical α -helix behaviour (a) –solid line– before adsorption, and (b) –dotted line– after release.

Adsorption isotherms

Peptide 1585 adsorption showed low adsorbed peptide solution concentration between A and B (as seen in figure 2), a small increase in adsorption with an attenuation of around 0.5 mmol/L between B and C. Peptide adsorption increased remarkably with concentration, producing a 230% increase in the 1.1 to 2.6 mmol/L range (as shown in D). Adsorption increased slowly between D and E for higher peptide concentrations. Above 3 mmol/L, the adsorbed amount increased even more due to possible peptide aggregation in experimental conditions.

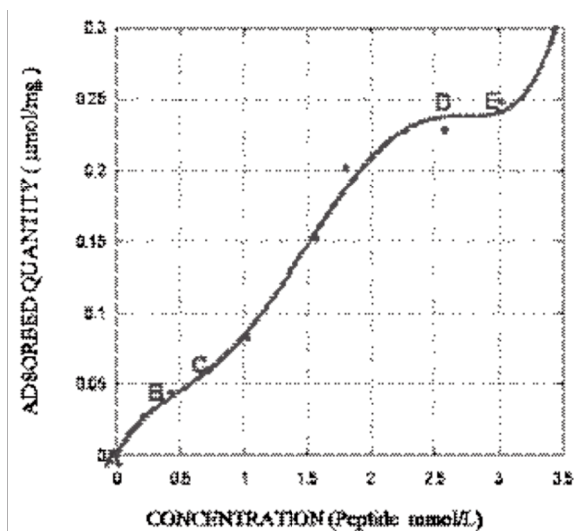


Figure 2. Isotherm for peptide 1585 adsorption on aluminium hydroxide.

This pattern showed that peptide molecules can cause reduced system entropy, producing a complex isotherm which could not be fully interpreted throughout the whole range of concentrations studied with Langmuir's model.

Initial AH saturation was produced to form the monolayer at concentrations below 0.5 mmol/L; then, a second arrangement of peptide molecules in solution occurred on the adsorbed molecules or double layer between 0.5 and 2.6 mmol/L. If such interpretation is correct, the isotherm can be separated into two independent concentration zones to apply Langmuir's model.

It was found that this model correctly interpreted peptide adsorption if Langmuir's equation (equation 1) applies to both areas, i.e. the area where the monolayer formation is supposed to occur, and in the 2 to 3 mmol/L range.

$$\frac{1}{m} = \frac{1}{bCm_n} + \frac{1}{m_n} \quad \text{Equation 1.}$$

In equation 1, m is the adsorbed amount of peptide ($\mu\text{mol/mg Al}$), b is the affinity constant L/mmol, C is the peptide concentration (mmol/L) and m_n is the adsorption capacity ($\mu\text{mol/mg of Al}$).

The adsorption capacity m_n was $0.086 \mu\text{mol/mg of Al}$ and the intercept b (2.057 L/mmol) was obtained by solving the monolayer slope. For the second layer, m_n was $0.359 \mu\text{mol/mg of Al}$, and 0.324 L/mmol for b . If adsorbed molecules in the first organisation

are found in condensate state on the solid surface; then it is evident that the amount of retained peptide m_n in the second organisation is higher, since adsorbed molecules in this concentration range come into contact with their own condensed phase, which would act as dissolvent in itself. The adsorption coefficient b in the first layer is higher as it measures direct peptide adsorption on the surface; whereas in the second layer, b represents part of the surface interaction, which can transcend adsorbed molecules, as well as the intermolecular interaction between adsorbed peptide, and that forming the double layer.

Such adsorption can be explained by the fact that peptides are complex molecules and their surface retention depends on their physical and chemical properties, but also by the aminoacids' position in the molecular mass sequence and tridimensional structure. Moreover, adsorption on AH is the final result of several molecular interactions and structural arrangements in the adsorbed layer.

NMR-determined peptide 1585 structure (22) has shown an alpha helix region ranging from aminoacid 4 - 12, and a random aminoacid 12-20 region in the helical wheel diagram shown in figure 3. It can be observed that most hydrophobic aminoacids are located on side 1; this molecule face will move away from the adsorbing surface since AH is in aqueous media and it does not contribute significantly to peptide adsorption. Adsorption will be considerably affected by charge repulsion, mainly by lysine (K6), when the other side of the helix is orientated towards the adsorbing surface, and because peptide and adsorbent net charges are positive (9.2 peptide 1585 isoelectric point and AH zero charge point 11 at physiological pH). It may thus be considered that if the peptide is orientated towards the surface by the non-structured region (where arginine (R) and lysine aminoacids (K) from positively-charged positions 13, 16 and 17 are found), there is a strong electrostatic repulsion with the surface, adsorption of peptide orientated in this way becoming negligible. Peptide interaction with the adsorbant would only be possible via glutamic acid (E) residues from negatively-charged positions 1 or 20, where an attractive interaction can appear. whose intensity depends on the balance of positive molecule charges and repulsion magnitude generated on the surface.

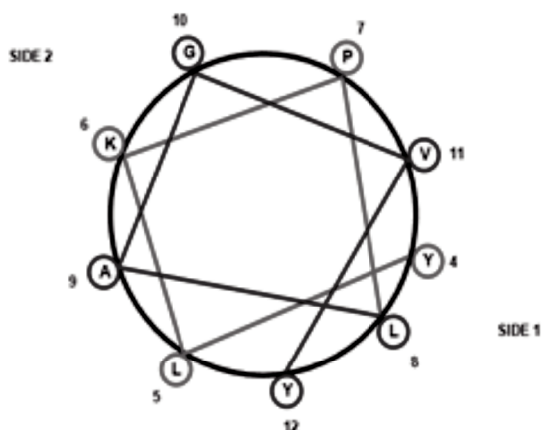


Figure 3. Peptide 1585 helical wheel. Distribution of aminoacid residues in both sides of the α -helix.

Equation 2 was used to prove if the adsorbate interaction with the surface occurs simultaneously via the glutamic acid residue of the amino and carboxy-terminal ends at two sites on the adsorbent, in the same way as it occurs in dissociative adsorption.

$$\frac{m}{m_n} = \frac{(bC)^{\frac{1}{2}}}{1 + (bC)^{\frac{1}{2}}} \quad \text{Equation 2.}$$

In equation 2, exponent $\frac{1}{2}$ implies two peptide contact points on the adsorbing surface.

The constants were calculated by the linearization of equation 2 thereby generating equation 3.

$$\frac{1}{m} = \frac{1}{m_n(bC)^{\frac{1}{2}}} + \frac{1}{m_n} \quad \text{Equation 3.}$$

This adsorption model did not interpret the observed data because the value of the intercept was negative, thereby implying monolayer capacity lacking physical sense.

This result confirmed that adsorption only occurs at one site (preferentially at the peptide amino-terminal end, where a glutamic acid (E1) residue is found) according to a monolayer formation molecular mechanism.

Adsorption model for the first organisation of the adsorbed layer.

The molecular docking (see figure 4a) determined the minimum retention distance of two peptide molecules on the adsorbant surface at nearby sites without attractive or repulsive interactions taking place (i.e. as in the Langmuir adsorption model). It establishes that when a peptide molecule approaches an already adsorbed molecule facing the helical region's hydrophilic sides (due to position 6 lysine), electrostatic and Van der Waals type repulsive interactions are caused with a 4.7×10^6 mV total energy, which decreased to 0 when the molecules were found at 3.7 nm mean distance.

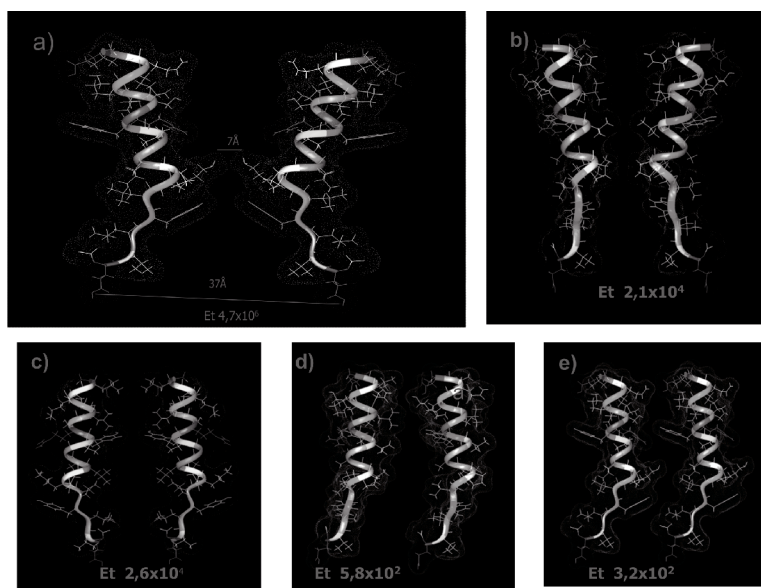


Figure 4. Peptide 1585 molecule α -helical approach to an adsorbed molecule; a) similar hydrophilic side approach; b) one to the front and the other behind; c) furthest α -helix hydrophobic sides faced with lysine residues; d) lysines orientating in parallel; e) lysine orientating in parallel towards the front, or in the same direction (but not in parallel).

Figure 4b shows that the total energy was 2.1×10^4 mV when lysines were orientated in parallel (one in front and the other behind) and became zero at 3.1 nm. If a molecule approached the furthest α -helix hydrophobic sides faced with lysine residues (K), as shown in figure 4c, Total Energy was 2.6×10^4 mV, and it decreased to zero when the intermolecular distance was 2.2 nm.

If a molecule approached in such a way that the lysine remained orientated in parallel towards the front or in the same direction (but not in parallel), as shown in figure 4d and 4e respectively, the total repulsion energy was 5.8×10^2 and 3.2×10^2 respectively, and it decreased to 0 at 1.8 nm intermolecular distance.

Taking into account this range of possibilities for the molecular adjustment at active adsorbant sites for first layer formation, it was assumed that the most probable interaction between two nearby molecules (A and B) would be the one implying the minimum energy change, and it would be caused at an intermolecular distance of 1.8 nm, orientating lysine side chains in the same direction and around each of them; three nearby molecules would be sited at 1.8 nm orientated in the same way regarding A and B. The adsorbed molecules would be sited at interactive sites separated by 1.8 nm for propagating this layer, keeping the described orientation, and leading to the minimum energy surface, as it is shown in figure 5a.

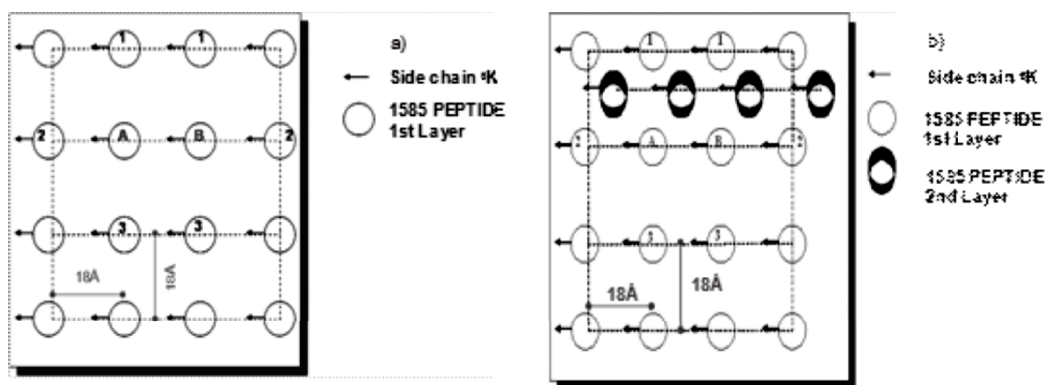


Figure 5. a) Adsorption model for the first molecular organisation of peptide 1585. b) Second layer formation in peptide adsorption.

Adsorption model for the second molecular organisation in the adsorbed layer

The second layer, which was formed by peptide 1585 by being adsorbed on retained molecules, is presented in the isotherm as it can be seen in figure 2. It can be assumed that when peptide molecules approach the adsorbed ones keeping the same orientation that was described for the first layer, a low-energy (-12 mV) interaction occurs (as determined by the docking software) between the carboxy-terminal of the positively-charged retained molecules (due to lysine in positions 16, 17), and the amino-terminal of those molecules forming the second layer as it is shown in figure 5b.

The excess of retained molecules that forms the second layer, regarding those from the first layer, means that others may be set in the interstices of the molecules separated at 1.8 nm. This can be established since the docking simulation with two

peptide molecules, which face the helix's hydrophobic side and in an anti-parallel orientation, presented low-energy interactions (-130 mV); which, thereby, enables the formation of dimer-type adsorbed molecular aggregates, as it is shown in figure 6.

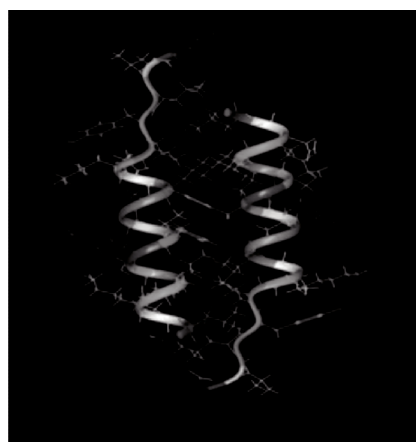


Figure 6. Dimer formation of peptide-adsorbed with low-energy interactions.

The proposed adsorption model for peptide 1585 retention on AH is in agreement with adsorption models known for having ionic tensoactive agents on charged surfaces (24-26).

As a peptide molecule can present areas with different polarity and a rather flexible tridimensional structure, it is possible that strong intermolecular interactions appear, inducing the formation of aggregates that have a certain degree of order as in the already-mentioned models. Some studies have shown evidence of α -helix peptide structure which is usually anphyathic, having some properties associated with tensoactive agents that can form micellar aggregates (26).

Peptide 1585 release

Release tests, as shown in figure 7, showed that the amount of peptide retained on hydroxide surface decreased remarkably for the first hours, and then it slowly decreased due to the competitive effect of the phosphate ion, preferentially adsorbed on AH, thereby displacing the peptide molecule.

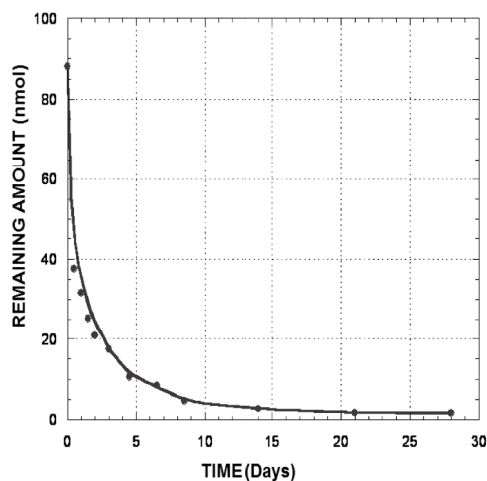


Figure 7. Amount of peptide retained on aluminium hydroxide by the phosphate ion effect.

This pattern can be described by the equation 4 (27).

$$C_R = C_o \times e^{-k_L t} \quad \text{Equation 4.}$$

In equation 4, C_R is the amount of remaining peptide (nmol), C_o is the total amount of adsorbed peptide at 310 K, k_L is the release constant (days^{-1}) and t is the time (days).

The following transformation (equation 5) was carried out to corroborate if this equation correctly

interpreted peptide 1585 desorption throughout all time ranges, and for determining the constants.

$$\ln C_R = \ln C_o - k_L t \quad \text{Equation 5.}$$

Thus, $\ln C_R = 3.82 - 0.39t$, with a correlation coefficient r^2 of 0.998, which was obtained by plotting the experimental data according to equation 5, where the total adsorbed concentration at 310 K was 45.83 μmol , and the release constant was 0.39 days^{-1} representing the peptide 1585 specific desorption rate.

By substituting the value of these constants in equations 4 and 5, the experimental data having dispersion no greater than 1.87% reproduced peptide release until day 2 when 76.27% of the total adsorbed peptide had been released at 310 K from a 1 mmol/L solution. The model did not comply for longer times since the desorbed peptide concentration in the environment remarkably increased the remaining adsorbed amount in an "in vitro" system, decreasing the release rate by diffusion effects, and thereby displacing equilibrium.

Results showed that peptide 1585 release at 310 K in the presence of the phosphate ion is a fast process governed by first order kinetics and limited to a two-day diffusion. This fact was not predictable for "in vivo" systems where release site may not be present, since the released substrate is processed for presentation to immune system cells.

If the result of adsorption at 298 K is compared to the one obtained at 310 K, then it is concluded that adsorption is affected by temperature, and reiterates the high release found at 310 K. If this result is correlated with the usual procedure, where the peptide is adsorbed at 298 K, conserved at 313 K, and inoculated at 310 K, then a patient will receive an almost instantaneous 57.36% peptide dose; while the controlled release at 310 K will occur for the remaining 42.64% concentration. This might be convenient since it raises the possibility of its recognition by immune system cells, but may cause unwanted allergic reactions.

The same calculations for peptide 1585 release with adsorptions performed with 1.5, 2.5 and 3.5 mmol/L solutions gave a similar pattern, thereby validating the proposed model.

The time taken for the 50% release of the remaining peptide ($t_{1/2}$) was calculated by only considering the time range for the peptide 1585

release (i.e. the controlled release), thereby generating equation 6.

$$t_{\frac{1}{2}} = \frac{\ln 2}{k_L} \quad \text{Equation 6.}$$

In equation 6, $t_{\frac{1}{2}}$ is the mean life time and k_L is the release constant. Table 1 summarizes the results, and it shows that $t_{\frac{1}{2}}$ increased with the amount of retained peptide for AH up to 3.15 days.

Table 1. Values for the constants corresponding to the kinetic analysis of peptide 1585 release.

Concentration (mmol/L)	r ²	C ₀ (μmol)	-k _L (days ⁻¹)	Validity interval (days)	t _{1/2} (days)
1	0.997	45.83	0.39	1-2	1.78
1.5	0.984	94.86	0.29	1-6	2.39
2.5	0.994	106.53	0.22	1-14	3.15
3.5	0.992	139.3	0.22	1-14	3.15

Adsorption reversibility

Results presented in table 2 and those illustrated in figure 1, curves (a) solid line and (b) dotted line, showed that the released peptide maintained its physicochemical and structural characteristics before adsorption and after their release. Results shown in table 3 allowed us to infer that peptide 1585 adsorption is totally reversible in the experimental conditions described here.

Table 2. Results of peptide 1585 characterisation by RP-HPLC and MALDI- TOF MS: (a) Before the adsorption and (b) after the release.

RP-HPLC Rt (min)		MALDI- TOF MS [M + H] ⁺ , (Da)	
(a)	(b)	(a)	(b)
23.6	23.1	2348.8	2348.1

Table 3. Percentages (%) of release peptide at 28 days.

Concentration for the adsorption (mmol/L)	Released peptide (%)
1.0	98.25
1.5	99.95
2.5	99.46
3.5	99.43

Peptide 1585 adsorption on AH generates complex isotherms, suggesting the formation of several layers around the adsorbent particles. This can be interpreted by applying Langmuir's model independently to the formation of each layer, while the adsorption coefficient is higher in the first layer and the adsorption capacity is higher in the second.

Adsorbed peptide release on AH in the presence of phosphate was performed according to first order kinetics, and it was limited by diffusion effects. Adsorption was totally reversible in the presence of the 4 mM phosphate ion, with pH 7.0 at 310 K, since desorbed molecules showed identical physicochemical characteristics to those of the initial peptide.

CONCLUSIONS

Peptide 1585 adsorption on AH generates complex isotherms, suggesting the formation of several layers around the adsorbent particles. This can be interpreted by applying Langmuir's model independently to the formation of each layer, with the adsorption coefficient being greater in the first layer and adsorption capacity greater in the second.

Adsorbed peptide release on AH in the presence of phosphate was performed according to a 1st order kinetic, which was limited by diffusion effects. Adsorption was totally reversible in the presence of the 4 mM phosphate ion, with pH 7.0 at 310 K, since desorbed molecules showed identical physicochemical characteristics to those of the initial peptide.

Even though the results obtained in this study cannot be extrapolated to an "in vivo" vaccination system with peptide molecules, this is the first time that adsorption, release and its reversibility have been physico-chemically characterised. Such parameters have an invaluable importance if they are used for formulating adsorbed peptide vaccines on a frequently used adjuvant like AH. The experience gained in this study draws the attention to the need for standardising physicochemical conditions for optimising the adsorption, which has a paramount importance in formulating a molecule for administering it and presenting it to the immune system.

ACKNOWLEDGMENTS

We would like to thank Universidad Nacional de Colombia for their financial support and Jason Garry for reading the manuscript.

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