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Simple model of the transduction of cell-penetrating peptides

K. Cahill

Biophysics Group, Department of Physics & Astronomy, University of New Mexico, Albuquerque, NM 87131, USA
E-mail: cahill@unm.edu

Abstract: Cell-penetrating peptides (CPPs) such as HIV's trans-activating transcriptional activator (TAT) and polyarginine rapidly pass through the plasma membranes of mammalian cells by an unknown mechanism called transduction. They may be medically useful when fused to well-chosen chains of fewer than about 35 amino acids. The author offers a simple model of transduction in which phosphatidylserines and CPPs effectively form two plates of a capacitor with a voltage sufficient to cause the formation of transient pores (electroporation). The model is consistent with experimental data on the transduction of oligoarginine into mouse C_2C_{12} myoblasts and makes three testable predictions.

1 Cell-penetrating peptides

Polyarginine, TAT [1, 2], penetratin and other short, positively charged peptides can penetrate the plasma membranes of live cells and can tow along with them cargos that greatly exceed the 600 Da restriction barrier. They are promising therapeutic tools when fused to well-chosen sequences of fewer than about 35 amino acids [3–15].

TAT carries cargos across cell membranes with high efficiency by at least two functionally distinct mechanisms according to whether the cargo is big or small [16]. Big cargos, such as proteins or quantum dots, enter via caveolae endocytosis and macropinocytosis [17, 18], and relatively few escape the cytoplasmic vesicles in which they then are trapped [16].

Small cargos, such as peptides of fewer than 30–40 amino acids, enter both slowly by endocytosis and rapidly by transduction, an unknown mechanism that uses the membrane potential [16, 19–21]. Peptides fused to TAT enter cells within seconds [22].

Section 2 reviews some facts about plasma membranes. Section 3 reviews how the phospholipid bilayer prevents ions from crossing the plasma membrane. Section 4 describes a simple model of transduction in which cell-penetrating peptides (CPPs) on the outer leaflet and phosphatidylserines (PS) on the inner leaflet form a kind of capacitor with a voltage sufficient to favour the formation of transient pores (electroporation). Section 5 shows that the model is consistent with measurements made by Tünnemann *et al.* [23] on the transduction into mouse myoblasts of oligoarginines and oligolysines carrying fluorophores of 400 Da. Section 6 tells how to test three predictions of the model. The paper ends with a short summary in Section 7.

2 Plasma membranes

The plasma membrane of a mammalian cell is a lipid bilayer that is 4 or 5 nm thick. Of the four main phospholipids in it, three – phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM) – are neutral, and one, PS, is negatively charged. In live cells, PE and PS are mostly in the cytosolic layer, and PC and SM in the outer layer [24, 25]. Aminophospholipid translocase (flippase) moves PE and PS to the inner layer; floppase slowly moves all phospholipids to the outer layer [24].

Glycolipids make up about 5% of the lipid molecules of the outer layer of a mammalian plasma membrane. Their hydrocarbon tails normally are saturated. Instead of a modified phosphate group, they are decorated with galactose, glucose, GalNAc = N-acetylgalactosamine, and other sugars. The most complex glycolipids – the

gangliosides – have negatively charged sialic-acid (NANA) groups. Incidentally, cholera toxin binds to and enters cells that display the G_{M1} ganglioside [25].

A living cell maintains an electrostatic potential of between 20 and 120 mV across its plasma membrane. The electric field E within the membrane points into the cell and is huge, about 15 mV/nm or 1.5×10^7 V/m if the potential difference is 60 mV across a membrane of 4 nm. Conventionally, one reports membrane potentials as the electric potential inside the cell minus that outside, so that here $\Delta V = -60$ mV. Near but outside the membrane, this electric field falls-off exponentially $E(r) = E \exp(-r/D_\ell)$ with the ratio of the distance r from the membrane to the Debye length D_ℓ, which is of the order of a nanometer. The rapid entry of TAT fused to peptides is frustrated only by agents that destroy the electric field E [16], which applies a force qE to a CPP of charge q.

Most of the phospholipids of the outer leaflet of the plasma membrane are neutral PCs & SMs. They vastly outnumber the negatively charged gangliosides, which are a subset of the glycolipids, which themselves amount only to 5% of the outer layer. Imagine now that CPP-cargo molecules are in the extra-cellular environment. Many of them will be pinned down by the electric field $E(r)$ just outside the membrane, their positively charged side-chains interacting with the negative phosphate groups of neutral dipolar PC & SM head groups. (Other CPP-cargo molecules will stick to negatively charged gangliosides and to glycosaminoglycans (GAGs) attached to transmembrane proteoglycans (PGs); these slowly will be endocytosed. Heparan-sulfate PGs are needed for TAT-protein endocytosis [26].) It is crucial that the dipolar PC & SM head groups are neutral and so do not cancel or reduce the positive electric charge of a CPP-cargo molecule. The net positive charge of a CPP-cargo molecule will attract negatively charged PSs and cause them to diffuse towards the part of the inner leaflet directly below the CPP-cargo molecule. This is the starting point for the model described in the next two sections.

3 Puzzle

The dielectric constant $\epsilon_\ell \simeq 2$ of the hydrocarbons of a lipid bilayer is much less than that of water $\epsilon_w \simeq 80$. Thus, the difference $\Delta E_{w\to\ell}$ in the electrostatic energy of an ion of charge q and effective radius a in the bilayer and in wate [27] is

$$\Delta E_{w\to\ell} = \frac{q^2}{8\pi\epsilon_0 a}\left(\frac{1}{\epsilon_\ell} - \frac{1}{\epsilon_w}\right) \tag{1}$$

or 3.5 eV if the ion's charge is that of the proton and its radius is $a = 1$Å. This energy barrier is far larger than the 0.06 eV gained when a unit charge crosses a 60 mV phospholipid bilayer. Thus, an ion will not cross a cell's plasma membrane unless a transporter or a channel facilitates (and regulates) its passage.

In the present model of CPP transduction, the electrostatics of the CPP-cargo complex and the role of PSs on the inner leaflet play key roles.

The electrostatics of a cationic polypeptide such as TAT or polyarginine are more complex than for an ion. I will model the CPP and its cargo in water as a sphere with its positive charges on its surface. The density of a protein of mass M kDa is estimated [28] to be

$$\rho(M) = (0.8491 + 0.0873\, e^{-M/13})\ \text{kDa/nm}^3 \tag{2}$$

A CPP-cargo complex would not be expected to fold as densely as a natural globular protein, and so for it the estimate $\rho(M)$ is more of an upper bound. The radius r of a putative sphere consisting of M kDa of CPP and cargo then would be at least

$$r \gtrsim \left(\frac{3}{4\pi}\frac{M}{\rho(M)}\right)^{1/3} \text{nm} \tag{3}$$

For instance, a CPP of N arginines and a tiny fluorophore cargo of 400 Da has a mass of $M_N = 0.1562N + 0.4$ kDa, and so its radius would satisfy

$$r \gtrsim \left(\frac{3}{4\pi}\frac{M_N}{\rho(M_N)}\right)^{1/3} \text{nm} \tag{4}$$

which gives $r = 0.75$ nm for $N = 8$ arginines. The lower bounds on the radii for $N = 5$–12 are listed in column 2 of the Table 1.

For larger cargos of $N_c = 50 - 100$ amino acids of 130 Da each, the lower bounds on the radii range from 1.25 to 1.59 nm. (In what follows, N_c will represent the number of amino acids in the cargo or the mass of the cargo in Daltons divided by 130 Da.) Adding another 0.8 nm for

Table 1 Radius r of a CPP–cargo molecule of N arginines and a cargo of 400 Da, its change in electrostatic energy $\Delta E_{w,\ell}$ when transferred from water to hydrocarbon (11), and the short-distance correction ΔE_{sdc} (12). Distances are in nanometres and energies in electron volts

N	r	$\Delta E_{w,\ell}$	ΔE_{sdc}
5	0.67	4.61	3.90
6	0.70	6.39	4.68
7	0.73	8.41	5.46
8	0.75	10.64	6.24
9	0.78	13.06	7.02
10	0.80	15.68	7.80
11	0.82	18.48	8.58
12	0.84	21.44	9.36

the PC/SM head groups extends these lower bounds on the radii to 2.05–2.39 nm. The diameters of these spheres would approach or exceed the normal 4.4 nm of the lipid bilayer. The extra energy penalty because of the distortion of the plasma membrane may explain why CPPs cannot transduce cargos of more than 50 amino acids [16].

If the CPP–cargo molecule were a charged conducting sphere of radius r and charge q, then its electrostatic energy in water would be

$$E(N, N_c, q, w) = \frac{q^2}{8\pi\epsilon_0\epsilon_w r} \tag{5}$$

This term neglects the short-distance detail of the electric field near the q/e positive unit charges e of the CPP–cargo molecule. So a short-distance correction term

$$E_{sdc}(N, N_c, q, w) = \frac{qe}{8\pi\epsilon_0\epsilon_w a} \tag{6}$$

proportional to q must be added to $E(N, N_c, q, \mathrm{w})$. The short distance a is an adjustable parameter, which should turn out to be several Å since the term E_{sdc} is a correction to be added to $E(N, N_c, q, w)$ and not the entire electrostatic energy.

As the CPP–cargo molecule enters the lipid bilayer, the phosphate groups of the PC and SM of the outer leaflet bind to the positively charged guanidinium and amine groups of the CPP. The positive charges of the phosphocholine groups of PC and SM are about $d = 5$ Å from their phosphate groups [29]. The binding of PC and SM therefore approximately increases the effective radius of the charged sphere to $r_m \simeq r + d$. The electrostatic energy of this complex in the hydrocarbon tails of the lipid bilayer then is

$$E(N, N_c, q, \ell) \simeq \frac{q^2}{8\pi\epsilon_0\epsilon_\ell(r+d)} \tag{7}$$

apart from a correction factor

$$E_{sdc}(N, N_c, q, \ell) = \frac{qe}{8\pi\epsilon_0\epsilon_\ell a} \tag{8}$$

similar to (6).

Apart from correction terms, the electrostatic energy penalty when the CPP–cargo molecule enters the lipid bilayer from water as a CPP–cargo–PC/SM complex is the difference

$$\Delta E^0_{w,\ell}(N, N_c, q) \simeq E(N, N_c, q, \ell) - E(N, N_c, q, \mathrm{w}) \approx \frac{q^2}{8\pi\epsilon_0\epsilon_\ell(r+d)}\left(1 - \frac{r+d}{r}\frac{\epsilon_\ell}{\epsilon_w}\right) \tag{9}$$

Because the thickness $t = 4.4$ nm of the lipid bilayer is only a few times that of the CPP–cargo–PC/SM complex, we also must include the Parsegian correction [27]

$$\Delta E_P = -\frac{q^2}{4\pi\epsilon_0\epsilon_\ell t}\ln\left(\frac{2\epsilon_w}{\epsilon_w + \epsilon_\ell}\right) \tag{10}$$

The sum of the water-to-lipid energy (9) and Parsegian's correction (10) is

$$\Delta E_{w,\ell}(N, N_c, q) = \Delta E^0_{w,\ell}(N, N_c, q) + \Delta E_P \tag{11}$$

The energy $\Delta E_{w,\ell}(N, N_c, q)$ is listed in column 3 of Table 1 for a CPP of $N = 5$–12 arginines towing a fluorophore cargo of 400 Da with $d = 0.5$ nm.

The short-distance correction terms augment this penalty by

$$\Delta E_{sdc}(N, N_c, q) = E_{sdc}(N, N_c, q, \ell) - E_{sdc}(N, N_c, q, w) = \frac{qe}{8\pi\epsilon_0\epsilon_\ell a}\left(1 - \frac{\epsilon_\ell}{\epsilon_w}\right) \tag{12}$$

and do not require Parsegian's correction because they are short-distance effects. This short-distance correction ΔE_{sdc} is listed in column 4 of Table 1 for a CPP of $N = 5$–12 arginines and a representative value of $a = 4.5$ Å for the short-distance parameter.

The net electrostatic energy penalty when the CPP–cargo molecule enters the lipid bilayer from water as a CPP–cargo–PC/SM complex is then the sum of (9, 10 and 12)

$$\Delta E_{w\to\ell} = \Delta E^0_{w,\ell} + \Delta E_P + \Delta E_{sdc} \tag{13}$$

A CPP of eight arginines carrying a fluorophore of 400 Da has a radius r of 0.75 nm, and with $a = 4.5$ Å, the change (13) in its electrostatic energy on going from water to lipid is

$$\Delta E_{w\to\ell}(8, 3, 8e) \simeq 16.9\,\mathrm{eV} \tag{14}$$

This energy barrier is 35 times bigger than the energy 0.48 eV that it gains by crossing a potential difference of 60 mV. So how and why does it cross?

4 CPP-PS capacitor and electroporation

My answer lies in the second part of the present model – the PSs. They comprise some 8–18% of the inner leaflet by weight [30]. The PSs diffuse laterally within that leaflet with a diffusion constant $D \simeq 10^{-8}\,\mathrm{cm}^2/\mathrm{s}$ [31] and so within 1 s spread to an area of 12 $\mu\mathrm{m}^2$, which is a significant fraction of the surface area of a eukaryotic cell. Attracted by the positive charge of two or more CPPs, several PSs cluster in the inner leaflet below the CPPs forming with them a kind of capacitor. In what follows,

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I will focus on the PSs because they are constrained to lie in the two-dimensional inner leaflet while other anions drift in the three-dimensional cytosol.

After oligoarginine CPPs are added to the extra-cellular fluid medium, the arginines of some of them will bind to the phosphate groups of the PC and SM phospholipids in the outer leaflet. The electrostatic potential of an isolated CPP may not be strong enough to form a pore because it is reduced at a point r in the lipid by the mean relative permittivity $\bar{\epsilon} = (\epsilon_w + \epsilon_\ell)/2$ to $V(r) = q/(4\pi\bar{\epsilon}r)$ and further reduced by counterions and image charges. But if a second CPP or a few CPPs should land near the first CPP and bind to PCs or SMs, then transduction becomes possible in the present model.

A CPP of N arginines can form an α-helix of length $L \simeq 0.16N$ nm (or a β-strand of length $0.34N$ nm). If two R^N CPPs lie a distance d apart bound to phosphate groups of the outer leaflet, then the charge $2Ne$ of the two CPPs is spread over approximately $L\ d$, giving a surface-charge density of $\sigma = 2Ne/(dL)$. The electric field of the two oligoarginines would attract $2N$ PSs to the part of the inner leaflet below the two R^Ns forming a kind of capacitor. If the voltage drop across this capacitor exceeds the threshold for the formation of the pre-pores or pores of electroporation, then the CPP and its cargo may enter the cell. This threshold is between ~250 and ~550 mV, depending upon the duration of the potential [32].

Due to its bidentate guanidinium group, arginine binds to phosphate groups better than lysine [21, 33]. Thus, oligoarginines would be more likely than oligolysines to bind to the phospholipids of the outer leaflet forming transiently stable capacitors with the PSs of the inner leaflet.

CPPs with $N = 12$ arginines can bind to more than one phosphate group, and so they can bind to more than one phospholipid of the outer leaflet. The number of such binding sites drops with the number of R's. In the 'picket-fence model', transmembrane proteins bind to actin and restrict the motion of outer-leaflet proteins and lipids [34]; so I assume that oligoarginines with $N > 8$ remain approximately stable for a few hundred milliseconds.

To include the effects of entropy and of the geometry of the oligoarginines (R^Ns) and the PSs, I wrote and ran a Monte Carlo program (included as supplementary material). In this code, I assumed that two R^Ns were parallel α-helices a distance d apart bound to phosphate groups of the outer leaflet. The PSs were allowed to move in two dimensions in the inner leaflet at sites r_k for $k = 1, \ldots, 2N$. The $2N$ positive charges of the two R^Ns are taken to lie fixed at the points

$$r^{\pm}_{j\mathrm{CPP}} = (\pm d/2,\ 0.16(N/2 - j),\ t) \tag{15}$$

in which the $\pm$-signs label the two R^Ns, and j labels the R's of the R^Ns ($0 \le j \le N$). The electrostatic energy of the PSs then is approximately

$$E = \frac{1}{4\pi\epsilon_0\bar{\epsilon}}\left(\sum_{i=2}^{N_{\mathrm{PS}}}\sum_{k=1}^{i-1}\frac{e^2}{|r_i - r_k|} - \sum_{k=1}^{N_{\mathrm{PS}}}\sum_{j=1}^{N}\frac{qe}{|r_i - r^{\pm}_{j\mathrm{CPP}}|}\right) \tag{16}$$

in which $\bar{\epsilon}$ is the mean permittivity

$$\bar{\epsilon} = \frac{1}{2}(\epsilon_w + \epsilon_\ell) \tag{17}$$

The Monte Carlo code used a simple Metropolis step in which the x–y coordinates of all the PSs were randomly varied, each by less than ± 0.025 nm. The code accepted any change in the positions of the PSs that lowered their energy E as given by (16) and accepted any change that raised their energy by ΔE conditionally with probability

$$P = e^{-\Delta E/(kT)} \tag{18}$$

in which k is Boltzmann's constant and T is 37°C. The simulation took place in a two-dimensional box with side $L = \sqrt{N/0.13}$ nm, and periodic boundary conditions were enforced to keep the number density of PSs constant at 13% within the box.

Each simulation started from a random configuration of PSs on the inner leaflet and ran for at least 10 million Metropolis steps. After at least 2 million steps for thermalisation, measurements of the positions of the PSs and of the electrostatic potential were taken every 1000 steps, and the positions of the PSs were recorded every 80 000 steps. The distance d separating the two CPPs was taken to be $d = 2$, 3 and 4 nm. (The simulations for $d = 3$ used ten times as many steps.)

The voltages were measured at the points (0, 0, t) and (0, 0, −0.1) nm. The offset by 0.1 nm was to avoid the chance arrival of a PS at the point (0, 0, 0). The voltage differences $\Delta V = V(0, 0, -0.1) - V(0, 0, t)$ are displayed in Table 2. At all three separations, CPPs with nine or more R's produce voltages that exceed the threshold (~250–~550 mV) for the formation of pre-pores or pores [32]. CPPs with ten or more R's may even exceed the threshold for irreversible electroporation.

The mean value of the distance of the PSs from the point (0, 0, 0) varied between $\langle r \rangle = 11.8$ nm for two R^5's separated by 2 nm and $\langle r \rangle = 27.5$ nm for two R^{12}'s separated by 4 nm.

In the present model, a CPP–PS capacitor increases the membrane potential sufficiently to cross the threshold for electroporation, at least for R^Ns with enough arginines. In its use of an electric field and of the binding of the CPPs to the phosphate groups of the phospholipids of the outer leaflet, the model has something in common with the adaptive-translocation model of Rothbard *et al.* [21]; in its use of neutral dipolar PC & SM head groups it is somewhat

Table 2 Voltage differences ΔV (mV) across the plasma membrane due to two R^N oligoarginines on the outer leaflet separated by a distance d nm and a flock of $2N$ PPs fluttering below them on the inner leaflet

N	ΔV $d = 2$ nm	ΔV $d = 3$ nm	ΔV $d = 4$ nm
5	−342	−229	−173
6	−398	−266	−198
7	−449	−300	−223
8	−504	−335	−248
9	−553	−369	−273
10	−601	−401	−297
11	−646	−433	−319
12	−687	−465	−340

similar to the work of Herce and Garcia [35] and of Tang *et al.* [36]. The key distinctive feature of the present model is the role of PSs in forming with the CPPs a CPP–PS capacitor with a voltage high enough to cause electropration.

5 Comparison with experiment

Tünnemann *et al.* [23] measured the ability of the L- and D-isoforms of oligolysine and of oligoarginine to carry fluorophores of ~400 Da into live cells. They found that oligoarginines transduced the fluorophores much better than oligolysines, that more arginines meant faster transduction, with L-R9 and L-R10 doing better than their shorter counterparts, and that the D-isoforms worked better than the L-isoforms, probably because of their greater resistance to proteases.

The present model is consistent with these experimental facts and explains them as follows: The oligoarginines crossed cell membranes more easily than the oligolysines because they were able to bind to the phosphate groups of the PCs and SMs in the outer leaflet; the oligolysines were not able to form a stable upper plate of the CPP–PS capacitor. CPPs with more arginines were transduced more rapidly because with more arginines they could bind to more PCs and SMs and because their higher charge led to a higher trans-membrane potential. The D-isoforms worked better than the L-isoforms because the capacitor mechanism is insensitive to the chirality of the amino acids and because proteases were less able to cut them.

This consistency of the capacitor model lends it some plausibility. The simplicity of the model suggests that it may apply to other oligocationic CPPs. But evolution finds what works, not what fits neatly into a model, and so other CPPs with different cargos may enter different cells by different mechanisms.

6 How to test this model

One way to test the model would be to compare the rates of polyarginine transduction in wild-type cells and in those that have little or no PS in their plasma membranes. If PS plays a key role as in the model of this paper, then the transduction of polyarginine fused to a cargo of less than 30 amino acids should be faster in the wild-type cells than in those without PS in their plasma membranes. Mammalian cell lines that are deficient in the synthesis of PS do exist [37–41], but they appear to have normal levels of PS in their plasma membranes [41]—presumably due to a lower rate of PS degradation [42].

Another test would be to construct artificial asymmetric bilayers [43–45] with and without PS on the 'cytosolic' side and to compare the rates of CPP-cargo transduction. If the present model is right, then the rate of transduction should be higher through membranes with PS on the cytosolic side than through membranes with no PS or with PS on both sides.

If CPPs do enter cells via molecular electroporation, then it may be possible to observe the formation of the transient pores by detecting changes in the conductance of the membrane [32]. Such measurements would be a third test of the model and would let one determine both whether CPP-transduction is related to the presence of PS on the cytosolic side of the membrane and whether it proceeds via molecular electroporation.

7 Summary

CPPs can carry into cells cargos with molecular weights of an order of magnitude greater than the nominal limit 500 of the 'rule of 5' [46]. Section 4 describes a model in which PSs play key roles in the transduction of CPP–cargo molecules. In this model, the positively charged CPP on the outer leaflet and the negatively charged PSs on the inner leaflet form a kind of capacitor with a trans-membrane potential sufficient for electroporation (−250 to −550 mV). The model is consistent with experimental data [23] on the transduction of the L- and D-isomers of oligoarginine and oligolysine CPPs into mouse myoblasts.

The model predicts that mammalian cells that lack PS in their plasma membranes transduce oligocations poorly, that artificial asymmetric bilayers with PS on the cytosolic side transduce oligocations better than ones without PS, and that transduction is accompanied by brief changes in the conductance of the membrane.

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```
 module defs
 integer, parameter :: i8b = selected_int_kind(18)
 integer, parameter :: i4b = selected_int_kind(9)
 integer, parameter :: i2b = selected_int_kind(4)
 integer, parameter :: ip = kind(1)
 integer, parameter :: sp = kind(1.0)
 integer, parameter :: dp = kind(1.0d0)
 integer, parameter :: qp = selected_real_kind(33,599)
 integer, parameter :: dc = kind((1.0d0,1.9d0))
 real(dp), parameter :: pi = 3.14159265358979323844_dp      &
 , twopi = 6.28318530717958647688_dp, eps = 0.01_dp                  &
 , twopiover3 = 2.09439510239319549229_dp                            &
 , sin37 = 0.59972214027798415249_dp &  ! sin(36.85)
 , cos37 = 0.80020831941463461536_dp &  ! cos(36.85)
 , cos106 = -0.28066670892078779654_dp  ! cos(106.3)
  real(dp) :: omegaalpha = pi, &                          ! 180 deg
              phialpha = -1.04719755119659774614_dp, &    ! -60 deg
              psialpha = -0.87266462599716478845_dp, &    ! -50 deg
              omegabeta = pi, &                           ! 180 deg
              phibeta  = -1.74532925199432957691_dp, &    !-100 deg
              psibeta  =  2.35619449019234492883_dp       ! 135 deg
! see ribosome.f90 for better values
! J Mol Bio(1996) 264, 1180 says 179.6 degrees is a better mean for omega.
  real(dp), dimension(9), parameter :: Idp = &
    (/ 1.0_dp, 0.0_dp, 0.0_dp, &
       0.0_dp, 1.0_dp, 0.0_dp, &
       0.0_dp, 0.0_dp, 1.0_dp /)
  integer(i4b), dimension(2), parameter :: shape = (/3, 3/)
  real(dp), dimension(3,3), parameter :: Id = reshape(Idp,shape)
 end module defs
```

```
program capmat ! Here I assume the PSs are on water-lipid interface.
  ! computes geometry of PSs under a CPP
  ! and the energy gained by the CPP in the middle of the bilayer.
  ! I use the simplest model possible.
  use defs; implicit none
  character(len=20)::outfile,imagefile
  integer(i4b)::N ! number of positive charges in CPP
  integer(i4b)::Np ! number of PSs
  integer(i4b)::i,j,k,ij,jk,ijk,Ntries,Ntherm,Nmax,nmeas,Ngap
  integer(i4b)::Nimages,imageGap,icount
  real(dp),allocatable,dimension(:,:)::duh,r,minr,dr
  real(dp),allocatable,dimension(:,:,:)::rs
  real(dp)::rand,Vtotubar,Vtotlbar,VdPSubar,VdPSlbar
  real(dp)::Q ! charge of CPP
  real(dp)::B
  real(dp)::E,Ep,Ec,minE,minEp,minEc,bigE,Epp,oldE ! energies
  real(dp)::Epsum,Ecsum,Esum,Vtotbar,sepnm,VdPSuSum,VdPSlSum
  real(dp)::tinyE,tinyEc,tinyEp,minEpp,Ecppps,minEcppps ! energies
  real(dp)::scale,rms,rik,ri,hn,dn,rho2,sep,beg,spot
  real(dp)::VdPS,VdOR,VdPSu,VdPSl,VdORu,VdORl ! voltages of PSs, ORs
  real(dp)::Vtotu,Vtotl,Vtot,xsum,ysum,Ecbar,Epbar,xrms,yrms,rrms
  real(dp)::h,hnm ! height of CPP
  real(dp)::dnm ! depth of point of lower potential
  real(dp)::V ! potential due to CPP
  real(dp),parameter::elec=1.60217653D-19 ! C e's charge SI units
  real(dp),parameter::epsl=2.0_dp ! lipid dielectric
  real(dp),parameter::epsw=78.0_dp ! water dielectric (at 37 C?)
  real(dp),parameter::eps0=8.854187817D-12 ! vacuum SI units
  real(dp),parameter::epsbar = 0.5d0*(epsw+epsl)
  real(dp),parameter::kB = 8.617343d-5 ! eV/K
  real(dp),parameter::Tb = 273.15d0 + 37.0d0 ! body temperature
  real(dp),parameter::beta = 1.d0/(kB*Tb)
  real(dp),parameter::d = 1.0d-10 ! 1 A depth of point of lower potential
  read(5,*); read(5,*) N, Np
  read(5,*); read(5,*) sepnm
  read(5,*); read(5,*) Ntherm, Ntries, Ngap, Nimages
  hnm = 2.2d0; dnm = 1.0d9*d
  imageGap = (Ntries - Ntherm)/Nimages
  h = hnm*1.0D-9; sep = sepnm*1.0d-9; oldE = 1.0d50
  read(5,*); read(5,*) outfile,imagefile
  Q = N; r = 0.0_dp; scale = 0.05_dp
  allocate(duh(2,Np),r(2,Np),minr(2,Np),dr(2,Np))
  allocate(rs(2,Np,Nimages))
  B = 0.5d0*sqrt(real(Np,dp)/0.13d0)*1.0d-9
  call random_seed()
  call random_number(duh)
  r = scale*(duh-0.5_dp)*3.0D-9 ! meters
  Ecsum = 0.0d0; Epsum = 0.0d0; xsum = 0.0d0; ysum = 0.0d0
  nmeas = 0; icount = 0
  do ij = 1, Ntries
     call random_number(duh)
     dr = scale*(duh - 0.5_dp)*1.0D-9; r = r + dr ! meters
     ! box condition
     do i = 1, 2
        do j = 1, Np
           if( r(i,j) > B ) then
              r(i,j) = r(i,j) - 2.0d0*B
           else if ( r(i,j) < - B ) then
              r(i,j) = r(i,j) + 2.0d0*B
           end if
        end do
     end do
     ! Find repulsive energy of PSs
```

```
    Ep = 0.0d0
    do i = 2, Np
       do k = 1, i-1
          rik = sqrt((r(1,k)-r(1,i))**2 + (r(2,k)-r(2,i))**2)
          Ep = Ep + 1.0_dp/rik
       end do
    end do
    Ep = Ep/epsbar
    ! Find energy due to CPP-PS interaction
    Ec = 0.0d0
    beg = (0.5d0*0.16d-9)*real(N,dp)
    do j = 1, N
       spot = beg - (0.16d-9)*real(j,dp)
       do i = 1, Np
          ri = sqrt((r(1,i)-sep)**2 + (r(2,i)-spot)**2 + (2.0d0*h)**2)
          Ec = Ec - 1.0d0/ri
          ri = sqrt((r(1,i)+sep)**2 + (r(2,i)-spot)**2 + (2.0d0*h)**2)
          Ec = Ec - 1.0d0/ri
       end do
    end do
    Ec = Ec/epsbar
    Ep = Ep*elec/(4.0_dp*pi*eps0); Ec = Ec*elec/(4.0_dp*pi*eps0)
    E = Ep + Ec
    if ( E < oldE ) then ! accept
       oldE = E
    else
       call random_number(rand)
       if ( rand <= exp(-beta*(E - oldE)) ) then ! accept
          oldE = E
       else ! reject
          r = r - dr
       end if
    end if
    if ( ij > Ntherm .and. mod(ij-Ntherm,Ngap) == 0 ) then ! measure
       nmeas = nmeas + 1
       Ecsum = Ecsum + Ec
       Epsum = Epsum + Ep
       xsum = xsum + dot_product(r(1,:),r(1,:))
       ysum = ysum + dot_product(r(2,:),r(2,:))
       ! Now compute the voltage at (0,0,2h) due to the PSs:
       VdPS = 0.0d0
       do i = 1, Np
          ri = sqrt( r(1,i)**2 + r(2,i)**2 + (2.0d0*h)**2)
          VdPS = VdPS - 1.0d0/ri
       end do
       VdPSu = VdPS*elec/(4.0_dp*pi*eps0*epsbar)
       VdPSuSum = VdPSuSum + VdPSu
       ! Now compute the voltage at (0,0,-d) due to the PSs:
       VdPS = 0.0d0
       do i = 1, Np
          ri = sqrt( r(1,i)**2 + r(2,i)**2 + d**2 )
          VdPS = VdPS - 1.0d0/ri
       end do
       VdPSl = VdPS*elec/(4.0_dp*pi*eps0*epsbar)
       VdPSlSum = VdPSlSum + VdPSl
    end if
    if ( ij > Ntherm .and. mod(ij-Ntherm,imageGap) == 0 ) then ! image
       icount = icount + 1
       do k = 1, Np
          rs(:,k,icount) = r(:,k)
       end do
    end if
 end do
```

```
  VdPSubar = VdPSuSum/real(nmeas,dp)
  VdPSlbar = VdPSlSum/real(nmeas,dp)
  ! Now compute the voltage at (0,0,2h) due to the R^Ns
  VdOR = 0.0d0
  beg = (0.5d0*0.16d-9)*real(N,dp)
  do j = 1, N
     spot = beg - (0.16d-9)*real(j,dp)
     ri = sqrt( sep**2 + spot**2 )
     VdOR = VdOR + 1.0d0/ri
  end do
  Vdor = 2*Vdor ! to count both R^Ns
  VdORu = VdOR*elec/(4.0_dp*pi*eps0*epsbar)
  ! Now compute the voltage at (0,0,-d) due to the R^Ns
  VdOR = 0.0d0
  do j = 1, N
     spot = beg - (0.16d-9)*real(j,dp)
     ri = sqrt( sep**2 + spot**2 + (2.0d0*h)**2 + d**2 )
     VdOR = VdOR + 1.0d0/ri
  end do
  Vdor = 2*Vdor ! to count both R^Ns
  VdORl = VdOR*elec/(4.0_dp*pi*eps0*epsbar)
  Vtotubar = VdPSubar + VdORu
  Vtotlbar = VdPSlbar + VdORl
  Vtotbar = Vtotlbar - Vtotubar
  Ecbar = Ecsum/real(nmeas,dp); Epbar = Epsum/real(nmeas,dp)
  xrms = sqrt(xsum/real(nmeas,dp)); yrms = sqrt(ysum/real(nmeas,dp))
  rrms = sqrt((xsum + ysum)/real(nmeas,dp))
  open(7,file=outfile)
  write(7,'(a,i3,a,f5.2,a,i3,a,f5.2,a,f5.2,a)')'For',N,' Rs @',2*hnm,' nm
&',&
       Np,' PSs  and a separation of 2*sep =',2*sep*1.0d9,' nm'
  write(7,'(a,i9,a,i9,a,i6,a,i6)')'after', Ntherm,' thermalization runs of',
&
       Ntries,' sweeps'
  write(7,'(3(a,i6))')'nmeas =',nmeas,' icount =',icount,' Nimages =',Nimages
  write(7,*)'<Ep> =',Epbar,' eV'
  write(7,*)'<Ec> =',Ecbar,' eV'
  write(7,'(3(a,f9.4))')'<x> =',xrms*1.d9,' <y> =',yrms*1.d9,' <r>
=',rrms*1.d9
  write(7,*)'<Vtotu> =',Vtotubar
  write(7,*)'<Vtotl> =',Vtotlbar
  write(7,*)'<Vtot> =',Vtotbar
  open(8,file=imagefile)
  do i = 1, Nimages
     do k = 1, Np
        write(8,*)1.d9*rs(1,k,i),1.d9*rs(2,k,i)
     end do
  end do
  close(8)
  close(7)
end program capmat
```