Structure and binding thermodynamics of viologen-phosphorous dendrimers to human serum albumin: A combined computational/experimental investigation

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Low-generation viologen-phosphorous dendrimers (VPDs) can be exploited as novel therapeutic agents, since they efficiently inhibit aggregation of amyloid-β into fibrils and are active against several strains of microorganisms. Human serum albumin (HSA), the most abundant plasma protein, is playing an increasing role as drug carrier in the clinical setting. Therefore, with the aim of exploiting HSA as a potential carrier for VPDs, in this work we performed a preliminary investigation of the interaction of six different VPDs 1–6 with HSA using a combined computational/experimental approach. First, different modeling techniques were employed to i) determine the dendrimer binding site on the HSA surface; ii) derive the free energy change $\Delta G_b$ involved in each dendrimer/HSA complex formation; iii) analyze in details all molecular determinants contributing to $\Delta G_b$, and iv) evaluate the eventual HSA structural variations induced by dendrimer binding. All modeling predictions were next validated using a series of experimental techniques, including isothermal titration calorimetry (ITC), circular dichroism (CD), and fluorescence quenching and decay. In aggregate, the results from this study allowed us to rank the affinity of the different viologen-phosphorous dendrimers 1–6 towards HSA and to formulate a molecular-based rationale for the differential binding thermodynamics of the resulting dendrimer/HSA complexes. According to our data, HSA can successfully and selectively bind VPDs 1–6, dendrimer 4 being the best cargo for this endogenous protein nanocarrier.

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1. Introduction

Human serum albumin (HSA) is the most abundant plasma protein (30–50 g/L human serum). With a molecular weight of 66.5 kDa and an average half-life of 19 days, this protein is synthesized in the liver (10–15 g/day). HSA is best known for its extraordinary ligand binding capacity, providing a depot for a wide variety of compounds that may thus be available in quantities well beyond their solubility in plasma.

HSA abundance makes it an important factor in the pharmacokinetic behavior of many natural metabolites (i.e., fatty acids, hormones, bilirubin, tryptophan, steroids, and metal ions) and of a plethora of therapeutic agents, affecting their efficacy and rate of delivery. This aspect has stimulated many efforts to understand what determined HSA/drug binding. Binding to HSA is also at the root of the development of contrast agents, for magnetic resonance imaging (MRI) endowed with high intravascular retention (blood pool agents), such as those used in the clinical practice for the visualization of vascular structures (magnetic resonance angiography) and for detecting regions with abnormal vascular permeability. In other cases, HSA holds some ligands in a strained orientation, providing their metabolic modifications, and renders potential toxins harmless by transporting them to disposal sites [1–3]. After acute hemolysis (e.g., after trauma or post-ischemic reperfusion), HSA binds heme that is released in the blood stream. Heme is then gradually transferred from HSA to human hemopexin, which allows its receptor-mediated re-uptake by parenchymal liver cells [4]. HSA also plays many other fundamental biological roles; among these, this protein i) is the essential regulator of blood osmotic pressure; ii) accounts for most of the antioxidant capacity of human serum, either directly or by binding and

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carrying radical scavengers, or by sequestering transition metal ions endowed with pro-oxidant activity; and iii) acts as a NO re-
pository and carrier, leading to covalent modification(s) of (macro)-
molecules [1].

Today, HSA is playing an increasing role as a drug carrier in the
clinical setting, via its coupling to low molecular weight drugs, or
its conjugation with bioactive protein/macromolecules, or the
encapsulation of drugs into albumin nanoparticles [5]. The under-
lying strategy is that binding of a therapeutic agent — either
covalently or physically - to human serum albumin might increase
its biodegradability, and its lack of toxicity and immunogenicity, e
specially uptake in tumor and inflamed tissues, its ready availability,
its biodegradability, and its lack of toxicity an immunogenicity
make it an ideal nanocarrier candidate for drug delivery.

Dendrimers undoubtedly constitute one of the most important
contributions of chemistry to nanoscience [6–9]. Their branched,
perfectly defined, layered, and three-dimensional architecture has
a size varying generally from 1 to less than 20 nm, depending on
the size (i.e., the number of self-similar layers). Independ-
dently of the chemical nature, a dendrimer structure is composed
of branches constituted of repeating units, emanating from a cen-
tral core. The extremities of the branches (also called terminal
units/groups) are generally reactive and can be functionalized at
will, depending on the properties that are targeted [10].

There are several main families of dendrimers, but new types of
dendritic structures are constantly being synthesized. One novel
class of dendritic compounds is viologen-phosphorous dendrimers
(VPDs). Phosphorus-containing dendrimers [11,12] are considered
to be particularly important for biomedical research because
phosphorus is essential for all terrestrial forms of life. The biological
properties of these compounds have been recently reviewed in
detail, including their positive effect on the growth of neuronal
cells, monocytes and natural killer cells, their anti-prion properties,
their use as delivery platforms for ocular drugs and transfection and
imaging agents, and their potential as highly sensitive biosensors
[13]. Used for a long time as unselective pesticides (aka Parquat),
viologen derivatives (4,4′-bipyridinium salts) can also exert severe
if not lethal poisoning in man [14]. The mechanism of viologen
toxicity involves the induction of generation of superoxide anions
and other reactive oxygen species (ROS), which ultimately result
into cell and tissue damage [15]. Moreover, there is increasing ev-
dence supporting the association of chronic Parquat exposure and
Parkinson’s disease [16].

Interesting, however, concomitant studies have shown that the
incorporation of viologen units as part of a dendrimeric structure
can yield new molecules with surprisingly beneficial biological activities
[17–19]. Thus, several cationic viologen-based dend-
rimers were tested for and found endowed with encouraging antiviral activity against human immunodeficiency virus (HIV-1),
herpes simplex virus (HSV), vesicular stomatitis, Punta Toro virus,
Sindbis virus, Rheovirus and Respiratory Syncytial virus (RSV) [20].
Recently, the group of Caminade and Majoral has synthesized a
novel series of dendrimers featuring both viologen units and
phosphorous groups in their structure [21], which differ in nature
of their core (tri- vs. hexa-functionalized), generation (G₀ vs. G₁),
number of viologen units, number and type of terminal groups
(-CHO vs. PO(Et)₂). Experiments in the field of neurodegenerative
diseases (e.g., transmissible spongiform encephalopathies, Alz-
heimer and Parkinson’s diseases) were carried out with these
viologen-phosphorous dendrimers (VPDs) [22]. Quite encour-
gagingly, all these VPDs, and specifically those bearing the
phosphate (PO(OEt)₃) terminal group, effectively inhibited
acetylcholinesterase and butyrlycholinesterase, two enzymes that
are implicated in Alzheimer’s disease as they co-localize with am-
yloid-β (Aβ) peptide plaques and accelerate the assembly of Aβ into fibrils [23]. More, these compounds also bind and influence the
activity of α-synuclein, another protein involved in the same
neurodegenerative pathology, thereby inhibiting its fibrillation
[24]. Another series of biological tests with the same VPDs con-
cerned their hemotoxicity, cytotoxicity, and antibacterial activity
[21]. It was shown that those compounds of generation 1 (i.e.,
bearing the highest number of positive charges) induced the
highest hemotoxicity (i.e., hemolysis); contextually, all VPDs were
found to be non-toxic towards B14 (healthy) cells but, at the same
time, very toxic towards cancerous N2a cells. In addition, most of
the tested VPDs exhibited good antimicrobial properties towards
S. aureus strain (Gram-positive bacteria) while the most-charged
dendrimers of the series also limited the growth of Gram-
negative bacterial strain (E. coli and P. vulgaris).

Notwithstanding the potential perspective of biomedical ap-
lications of viologen-phosphorous dendrimers outlined above,
quite surprisingly no studies beyond basic biological properties
(mainly cytotoxicity assays) of VPDs have been published so far.
Since the possible exploitation of viologen-phosphorous dendrimers
as nanomedicines proves to requires a deeper knowledge of their
biological behavior, in this work we decided to investigate the
interaction of VPDs with human serum albumin. As outlined above,
VPDs are able to interact with several, different human proteins;
thus, we reasoned that cationic viologen-phosphorous dendrimers
could exhibit a natural affinity for HSA, the most abundant and
highly negatively charged plasma protein. Should this hypothesis
be verified, the drug carrier native propensity of HSA could further
be exploited as generate a novel endogenous nanocarrier/cargo
system for the VPDs. Accordingly, we performed a preliminary
investigation of the interaction of six different VPDs with HSA using
a combined computational/experimental approach. First, different
modeling techniques were employed to i) determine the dendrimer
binding site on the HSA surface; ii) derive the free energy change
ΔG₀ involved in each dendrimer/HSA complex formation; iii)
analyze in details all molecular determinants contributing to ΔG₀,
and iv) evaluate the eventual HSA structural variations induced by
dendrimer binding. All modeling predictions were next validated
using a series of experimental techniques, including isothermal
titration calorimetry (ITC), circular dichroism (CD), and fluores-
cence quenching and decay. In aggregate, the results from this
study allowed us to rank the affinity of the different viologen-
phosphorous dendrimers towards HSA and to formulate a
molecular-based rationale for the differential binding thermody-
namics of the resulting dendrimer/HSA complexes.

2. Materials and methods

All chemicals were purchased from Sigma Aldrich and Acros
Organics, and used without further purification. Human serum al-
bumin (fatty acid free) was also purchased from Sigma and used as
supplied. The dendrimer cores trihydrazidophosphate sulfide
SP(NMeNH₂)₃ and hexahydrazido cyclophosphazene
N₃P₉(NMeNH₂)₆ were prepared following literature indications
[25].¹H and ¹³C NMR spectra of the dendrimers were recorded at
room temperature with a 500 MHz Varian NMR spectrophotom-
eter, using SiMe₄ as internal standard for NMR chemical shifts.
The following notation is adopted for NMR splitting patterns: s, singlet;
d, doublet; t, triplet; q, quartet; m, multiplet. Fourier-transformed
infrared spectra (FT-IR) were recorded on neat samples using a
Perkin–Elmer Spectrum RXI. Mass spectrometry (ESI-MS, positive
mode) was carried out with a Perkin–Elmer PE-API I Spectrometer.
2.1. Synthesis of viologen-phosphorous dendrimers

Six viologen-phosphorous dendrimers 1–6 with different end groups and number of viologen units were synthesized and studied in this work. Their chemical structures are shown in Fig. 1 while their main characteristics are listed in Table 1. All dendrimers were synthesized according to the procedure described by Ciepluch et al. [21]. The synthetic pathways and NMR characterization of all dendrimer molecules are reported in the Supporting Information.

2.2. Fluorescence quenching

All steady-state fluorescence spectra were recorded using a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Japan) equipped with a thermostatic cell holder and a 1 cm path length cell cuvette. Fluorescence quenching experiments were carried out by fixing HSA concentration (PBS buffer, pH 7.4) to $5 \times 10^{-6}$ mol/L whereas dendrimer concentration was varied in the range $0–20 \times 10^{-6}$ mol/L. Measurements were performed at 298, 303, and 310 K. An excitation wavelength of 290 nm was used and emission spectra were recorded from 300 to 440 nm. The excitation and emission slits were set to 5 nm and 10 nm, respectively. Time-resolved fluorescence decays were determined by the time-correlated single-photon counting (TCSPC) spectrometer on a Horiba Jobin Yvon Fluoromax4 spectrophotometer, using a picosecond diode laser (nanoLED-07) as the light source at 295 nm and TBX-04 detector (both from IBH, U.K.). The observed fluorescence transients were fitted using a nonlinear least-square fitting procedure [26].

2.3. Circular dichroism (CD) spectra

CD measurements of PBS buffered solutions of HSA in the absence and presence of VPDs 1–6 were carried out on a Jasco Corporation J-815 CD spectrophotometer (Jasco, USA) using a quartz cuvette of path length 1.0 cm at 1 nm pitch intervals. All CD spectra were recorded in the wavelength range 200–250 nm. The spectrophotometer was continuously purged prior and during the experiments with 99.9% nitrogen. Spectra were collected at scan speed of 50 nm/min with response time of 1 s at 298 K. Each spectrum was baseline corrected and the final plot was taken as an average of four accumulated plots. Results were expressed as mean residue ellipticity MRE, calculated according to the formula: $\text{MRE} = (\text{MRE}_{\text{obs}}/10) \times (\text{MMR}/L\text{C}_p)$, where MREobs is the observed ellipticity (mdeg), MMR is the mean residue molecular mass, L is the optical path length (cm), and C_p is the protein concentration (mol/L). To calculate the composition of the secondary structure of the protein [27], the SELCON3 [28], CONTIN [29], and CDSSTR programs [30] – as implemented in the CDPro software package - were used to analyze far-UV CD spectra. Final results were assumed when data generated from all programs showed convergence [31].

2.4. Isothermal titration calorimetry (ITC)

ITC experiments were conducted using a Nano ITC Technology (TA Instruments). Binding conditions were optimized for each viologen-phosphorous dendrimer. All experiments were performed in PBS buffer at pH 7.4. All solutions and buffers used in the experiments were degassed for 30 min at room temperature under stirring at 600 rpm prior to experiment. Upon filling cell and syringe, stirring was turned and the each system was allowed to thermally equilibrate for 1 h. For background correction, buffer (in cell) was titrated with the injection of dendrimers 1–6 at the same concentration; background was then subtracted from final curves. The binding and thermodynamic parameters: constant of binding ($K_b$), the number of binding centers per one molecule ($n$), and enthalpy of binding ($\Delta H_b$) were computed from actual calorimetric data by a non-linear fitting using the ITC software Origin. Using the fit output of $\Delta H_b$ and $K_b$, the free energy of binding $\Delta G_b$ and its entropic component $-T \Delta S_b$ were simply determined using standard thermodynamic relationships (i.e., $\Delta G_b = -RT \ln K_b$ and $-\Delta T \Delta S_b = \Delta G_b - \Delta H_b$). Statistics were performed on the thermodynamic parameters with a desired confidence interval of 95%. Each experiment was repeated at least in triplicate, and the average or multiple runs was used to obtain $K_b$.

2.5. Molecular simulations

2.5.1. Model building, refinement and simulation details

All simulations discussed in this work were carried out using the AMBER14 suite of programs [32] and performed with the GPU version of pmemd (pmemd.cuda) in AMBER14 on the EURORA GPU-CPU supercomputer (CINECA, Bologna, Italy). The entire MD simulation and data analysis procedure was optimized by integrating Amber14 in modeFRONTIER, a multidisciplinary and multi-objective optimization and design environment [33]. The six dendrimer models were built, parameterized and refined following a consolidated procedure described in details in our previous works [34–41]. Briefly, the 3D structure of each dendrimer was built and geometry-optimized using the Antechamber module of AMBER14.
consistently with General Amber Force Field (GAFF) [42]. Eventual missing force field terms were derived from quantum mechanical (QM) calculations using GAMESS software [43,44] and the paramfit feature of AMBER14. For QM calculations, the MP2/HF/6–31G level of theory was used. Partial charges were obtained via the RESP program implemented in AMBER14.

The x-ray available crystal structure of ligand-free human serum albumin (PDB code 1AO6.pdb) [45] was taken as starting point for protein model preparation. The structures of each dendrimer and that of the HSA were immersed in a box of TIP3P water molecules [46]. The dimension of each simulation box was chosen in order to ensure a 1 nm solvation shell around each solute structure. Suitable amounts of Na\(^+\) and Cl\(^-\) ions required to achieve solution neutrality and to realize a physiological ionic strength of 0.15 M were added to each system. The resulting hydrated structures were then subjected to an initial Steepest Descent (SD)/Conjugated Gradient (CG) minimization with 5.0 kcal/(mol\(\cdot\)Å\(^2\)) restraint on the solvent (solvent relaxation), followed by another round of CG minimization without restraints in order to eliminate all bad contacts between water molecules and the dendrimer/HSA structure.

Next, each minimized structure was subjected to molecular dynamics (MD) simulations in the canonical ensemble (constant volume/constant temperature, or NVT). During these 100 ps of MD, each system was gradually heated and relaxed to 300 K. The SHAKE algorithm [47] was applied to all covalent bonds involving hydrogen atoms. An integration time step of 2 fs was adopted together with the Langevin thermostat for temperature regulation (collision frequency = 2.0 ps \(^{-1}\)) [48]. The final heating step was followed by 50 ns of MD equilibration in the isochoric/isothermal (NPT) ensemble. Pressure control was exerted by coupling the system to a Berendsen barostat (pressure relaxation time 2 ps) [49]. The particle Mesh Ewald (PME) method [50] was used to treat long-range electrostatic interactions under periodic conditions with a direct space cut-off of 10 Å. A frame from each equilibrated MD trajectory of the dendrimers and HSA was extracted to build the different protein/dendrimer complex initial configuration.

For the construction of the dendrimer/HSA complex models, we resorted to Steered Molecular Dynamics (SMD) simulations [35,51–56]. Specifically, the dendrimer and HSA structures extracted from the corresponding equilibrated MD simulations were placed 60 Å away from each other in a solvated box. Next, the dendrimer was pulled close to its target protein by using a force of 50 kcal/(mol\(\cdot\)Å\(^2\)) and a velocity of 5 Å/ns. The backbone atoms of HSA were forced in their position by applying a weak restraint of 0.5 kcal/(mol\(\cdot\)Å\(^2\)). This allowed avoiding substantial deformation of the protein during the dendrimer pulling process. Once the dendrimer reached the proximity of HSA (i.e., distance between the dendrimer and HSA center of mass approximately 12 Å), this restraint was released and the both molecules were allowed to move to reach the final complex configuration.

Each resulting dendrimer/HSA complex was again equilibrated for 50 ns in the NPT ensemble and, starting from the last equilibrated frame, further 50 ns of simulation in an NVT ensemble were performed for structural and binding thermodynamic data collection and analysis.

2.5.2. Structural analysis

The structural analysis of the HSA in complex with the dendrimers was carried using the cpptraj program of AMBER14 and in-house developed python scripts. If not differently stated, all structural data presented in this work represent values averaged over the last 40 ns of the production runs, with MD trajectory snapshots taken every 40 ps.

2.5.3. Free energy of binding calculations

The free energy of binding \(\Delta G_b\) between each dendrimer and HSA was derived by applying our thoroughly validated methodology [34–41] based on the Molecular Mechanics/Poisson Boltzmann Surface Area (MM/PBSA) approach [57]. This computational technique employs snapshots taken from MD trajectories to estimate the average interaction energies based on the solute molecular mechanics internal energy (\(\Delta G_{\text{MM}}\)) and solvation energy (\(\Delta G_{\text{solv}}\)), this last obtained via Poisson–Boltzmann (PB) continuum solvent calculations. According to MM/PBSA, the overall binding energy \(\Delta G_b\) is given by the difference in energy between the HSA/dendrimer complex and the individual dendrimer and HSA molecules:

\[
\Delta G_b = \Delta G_{\text{complex}} - \Delta G_{\text{dendrimer}} - \Delta G_{\text{HSA}}
\]

where:

\[
\Delta G_b = \Delta G_{\text{MM}} + \Delta G_{\text{solv}} - T \Delta S_{\text{bind}}
\]

\[
\Delta G_{\text{MM}} = \Delta G_{\text{vdW}} + \Delta G_{\text{Coul}}
\]

\[
\Delta G_{\text{solv}} = \Delta G_{\text{gb}} + \Delta G_{\text{np}}
\]

\(\Delta G_{\text{MM}}\) is the system change in molecular mechanical energy upon binding, which consists of coulombic (\(\Delta G_{\text{Coul}}\)) and van der Waals (\(\Delta G_{\text{vdW}}\)) contributions, respectively. The solvation energy term \(\Delta G_{\text{solv}}\) consists of two components: the electrostatic term \(\Delta G_{\text{gb}}\) and the nonpolar term \(\Delta G_{\text{np}}\), respectively. \(\Delta G_{\text{np}}\) is obtained by solving the Poisson–Boltzmann equation [58] while \(\Delta G_{\text{gb}}\) can be obtained via the semi-empirical expression [59]:

\[
\Delta G_{\text{gb}} = \gamma \times \text{SASA} + \beta, \text{ in which SASA is the solvent accessible surface area of the molecule, } \gamma \text{ is the surface tension parameter (0.00542 kcal/Å}^2/mol), \text{ and } \beta = 0.92 \text{ kcal/mol. Finally, the entropic contribution } -T \Delta S_{\text{gb}} \text{ is calculated via normal mode of harmonic frequencies [60] obtained from a subset of minimized snapshots taken from the corresponding MD trajectories.}

The analysis of the energy of interaction between HSA and the dendrimers was accomplished with the MMPBSA.py script implemented in AmberTools14. Energy values were averaged over 200 frames taken during equally spaced time interval during the last 15 ns of the MD production steps. Normal mode analysis was carried out on a subset of 15 minimized MD snapshots even extracted from the relevant trajectory time frame used for energy calculations.
Finally, the effective type number of dendrimer residues and protein amino acids involved in binding, and the corresponding contribution to the binding free energy were obtained performing a per residue binding free energy decomposition exploiting the MD trajectories of each given HSA/dendrimer ensemble [61]. This analysis was carried out using the MM/GBSA approach [62], and was based on the same snapshots used in the binding free energy calculations.

3. Results and discussion

3.1. Determination of viologen-phosphorous dendrimer binding site on HSA

From structural standpoint, the countless solved x-ray crystal structures of HSA (per se or in complex with a myriad of metabolites/drugs) revealed that the protein, a 585 amino acid residue monomer, contains three homologous \( \alpha \)-helical domains, named I, II, and III, respectively (see Fig. 2). The domains each contain ten helices, divided into six-helix and four-helix subdomains (A and B) (see Fig. 2); the first four helices of A and B form similar anti-parallel \( \alpha \)-helix bundles.

To determine the most probable binding site for the viologen-phosphorous dendrimers 1–6 on the human serum albumin, we applied a validated procedure based on Steered Molecular Dynamics (SMD) simulations [35,51–56]. To the purpose, the protein was divided into three major regions (domains 1–3, Fig. 2, left panel); then, docking poses for all dendrimers where searched by SMD over these protein regions 1, 2, and 3. Furthermore, given the dimensions of some dendrimeric molecules, two further protein zones, encompassing regions 1–2 and 2–3, respectively, where further considering for SMD-guided docking operations (Fig. 2, right panel).

Calculations of the free energy of binding \( \Delta G_{\text{bind}} \) for each of the 5 resulting binding poses at the end of the corresponding SMD simulation (last time frame of Fig. 2, right panel) revealed a remarkable preference for binding of all these dendrimers to HSA in the 1–2 region (Table S1). Accordingly, all other possible binding modes were discarded and the detail thermodynamic analysis of the HSA/dendrimer binding was carried out using the most favorable complex configuration for each HSA/dendrimer couple only.

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**Fig. 2.** (Top left panel) Equilibrate molecular dynamics structure of HSA, in ribbon representation colored according to the different protein subdomains: IA [residues 5–107], sky blue; IB [108–196], dark slate blue; IIA [197–297], light green; IIB [298–383], sea green; IIIA [384–407], tan; IIIB [498–582], sandy brown. Water molecules are portrayed as white spheres; some Cl\(^{-}\) and Na\(^{+}\) ions and counterions are shown as big light gray spheres and small dim gray spheres, respectively. (Bottom left panel) Structure of HSA showing the three different protein regions selected for the viologen-phosphorous binding site determination via SMD: region 1 (residues 5–210, dark cyan); region 2 (residues 211–403, navy blue); region 3 (residues 404–582, lime green). (Right panel) Steered molecular dynamics (SMD) simulations to mimic the binding process of dendrimer 5 to the HSA onto the 5 protein regions 1, 2, 3, 1–2 and 1–3 (see text for details). The dendrimer is portrayed as red spheres while the HSA is in ribbon representation (coloring scheme as in bottom left panel). Water molecules, ions and counterions are omitted for clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.2. Thermodynamics of binding of viologen-phosphorous dendrimers 1–6 with HSA

The results of the application of the MM-PBSA calculations to the binding of VPDs 1–6 to HSA are presented in Table 2. Several considerations can be drawn from the analysis of this Table. First, all dendrimers are endowed with high affinity for human serum albumin, the predicted $K_d$ values ranging in the interval $10^4$–$10^6$ L/mol (Table 2, last column). However, the adopted simulation protocol also clearly ranks these dendrimers with respect to their protein binding capacity $\Delta G_{b}$ in the following order: $4 > 6 > 5 > 3 > 2 > 1$ (Table 2, 4th column), the best HSA binder being dendrimer 4 ($\Delta G_{b} = -13.40$ kcal/mol) and dendrimer 1 being the least effective ligand ($\Delta G_{b} = -9.03$ kcal/mol). From a general perspective, it appears that the presence of the -PO(OEt)$_2$ substituent as the dendrimer terminal group is more beneficial than the -CHO group to HSA binding. Indeed, irrespective of the dendrimer core, overall charge and generation, those dendrimers featuring the phosphonate group (i.e., 3–6) are all characterized by substantially more favorable values of $\Delta G_{b}$ (Table 2). However, somewhat contrary to expectations, within this latter subgroup the molecules of higher generation 5 and 6, bearing a larger number of positive charges on their outer shells, are predicted to be less effective than the lower generation counterpart 4 in binding HSA. Finally, the nature of the dendrimer core ($S=P$ vs $N_5P_3$), resulting in different multiplication of the dendrimer branches (3 vs. 6), seems to play a role in fostering protein/dendrimer binding, since, considering again the subgroup of dendrimers having the same terminal group (i.e., -PO(OEt)$_2$), lower (i.e., less negative) $\Delta G_{b}$ values can be found (Figure S11) for those molecules featuring the $S=P$ core (3 and 5) with respect to those (4 and 6) having $N_5P_3$ at their focal point (vide infra and SI for more discussion on these issues).

To gain further insight into the variegated affinity of dendrimers 1–6 for HSA, each global free energy of binding value was decomposed into its enthalpic ($\Delta H_{b}$) and entropic ($-\Delta S_{b}$) components, as shown in the second and third columns of Table 2, from which a typical enthalpy-entropy compensation behavior is inferred (Fig. 3, left panel). In fact, for all viologen-phosphorous dendrimers considered the enthalpic change is always favorable ($\Delta H_{b} < 0$) whereas entropy variation opposes binding ($-\Delta S_{b} > 0$). However, the entropic penalty paid by each dendrimer upon protein binding is always largely overwhelmed by the corresponding enthalpic gain (Fig. 3, right panel), thereby confirming the enthalpic-driven nature of the binding mechanism of dendrimers 1–6 to human serum albumin.

Further deconvolution of $\Delta G_{b}$ values into its components (Eqs. (2)–(4)) reveals the overall enthalpic contribution governing the spontaneous binding of all viologen-dendrimers 1–6 onto HSA is dominated by electrostatic interactions, as expected given the highly positively-charged nature of the dendrimers and the highly-negatively charged protein (–15e, as calculated from the amino acid composition). Specifically, the mean value of the total electrostatic energy contribution to dendrimer/protein binding ($\Delta G_{ele} = \Delta E_{coul} + \Delta G_{PB}$) is negative in all cases, and substantially more favorable than the corresponding value of the dispersive forces (i.e., van der Waals and hydrophobic interaction energies, $\Delta G_{disp} = \Delta E_{vdw} + \Delta G_{GPB}$), as shown in the left panel of Fig. 4.

Interestingly, however, the behavior of $\Delta G_{ele}$ is not monotonically dependent on the dendrimer charge: indeed, as highlighted in the right panel of Fig. 4, those dendrimers of higher generation, which bear a higher number of positively charged groups (i.e., 5 and 6), are only slightly more (if not less) effective in exploiting their charges upon binding HSA than their lower charge-bearing counterparts (i.e., 3 and 4). This sort of less-is-more effect [63] is not unusual in dendrimer/biomacromolecule association [34–41], and can be rationalized in terms of the capacity of each dendrimERIC structure to organize each single branch for optimal receptor binding, as discussed in detail in the following paragraphs.

To substantiate the effects exerted by the different dendrimer characteristics (i.e., core, charge, generation and terminal groups) on HSA binding affinity, we next processed data collected during equilibrated MD simulations of the single molecular species in the framework of the MM/PBSA theory. Specifically, we assessed the values of $\Delta G_{b,terminal}$ and $\Delta G_{b,protein}$ that is, the contribution to dendrimer/protein binding free energy yielded by each dendrimer branch and protein residues respectively (Fig. 5). To this aim, each dendrimer molecule has been subdivided into chemically consistent fragments, as shown in the left panel of Fig. 5. The right panel of Fig. 5 shows equilibrated molecular dynamics snapshots of the corresponding dendrimer/HSA complexes in which the protein residues involved in binding are highlighted.

The results of this complex analysis are shown in Fig. 6 A–D. From Fig. 6 A and B (and Tables S12-3) it can be seen that, independently of the dendrimer core, the most favorable contributions to HSA binding stem from the external double-positive viologen fragments (BPF) and from the dendrimer terminal groups (BZA/DEP). In fact, for each dendrimer, the sum of the BPF and BZA/DEP contributions - corresponding to $\sim 3.94$, $\sim 5.15$, and $\sim 4.62$ kcal/mol for the $S=P$ core-based dendrimers 1, 3, and 5, and to $\sim 4.488$, $\sim 7.066$, and $\sim 5.128$ kcal/mol for the $N_5P_3$ core-based dendrimers 2, 4, and 6, respectively (Tables S12–3) – amount, on average, to 85% of the total binding free energy afforded by all dendrimer fragments ($\Sigma \Delta G_{b,terminal}$ in Tables S12-3). Moreover, the presence of the -PO(OEt)$_2$ moiety (DEP) on the outer dendrimer shell is more beneficial to protein binding than the -CHO group (BZA). In fact, the synergistic combination of -PO(OEt)$_2$ structural flexibility, essential in maximizing dendrimer/protein contacts, and higher electronegativity, which potentiates the effect of the positive charges on the linked BPF fragments (Fig. 6A–B), renders the BPF/DEP sequence more effective than the BPF-BZA one. Focusing the attention of those molecules featuring the -PO(OEt)$_2$ terminal group (3–6), Fig. 6A–B also reveal that increasing the number of charged viologen branches in the dendrimERIC structure does not result in a corresponding increasing dendrimer affinity for HSA. In fact, by comparing dendrimers 3 and 5 (or 4 and 6), we see that the higher level of dendrimer structural complexity ultimately results in a negligible increase (3 vs. 5) if not in a decrease (4 vs. 6) of the dendrimer ability and efficacy in protein binding (i.e., less is more).

Panels C and D in Fig. 6 show the same analysis performed on the negatively charged HSA residues mainly involved in binding dendrimers 1–6 (see also Tables S14 and S15). As it can be evinced from this figure, the presence of a larger core ($N_5P_3$), with a multiplicity $N_i$ of 6, leads to dendrimers which exchange a higher number of contacts with the protein than in the case of dendrimers featuring the $S=P$ core ($N_i = 3$). This, in turn, results in a more overall favorable contribution to binding. In fact, the total contribution afforded by the HSA glutamic and aspartic acid residues in the case of dendrimers 1, 3 and 5 ($S=P$ core) is equal to $-2.509,$
Fig. 3. (Left) Decomposition of the free energy of binding $\Delta G_0$ of dendrimers 1–6 on HSA into its enthalpic ($\Delta H_0$) and entropic ($-T\Delta S_0$) contributions at 298 K. (Right) $-T\Delta S_0$ vs. $\Delta H_0$ scatter plot for the binding of dendrimers 1–6 to HSA.

Fig. 4. (Left) Decomposition of the binding enthalpy $\Delta H_0$ of dendrimers 1–6 in complex with HSA into overall contributions from electrostatic ($\Delta G_{ele} = \Delta E_{Coul} + \Delta E_{PB}$) and dispersive ($\Delta G_{disp} = \Delta E_{VdW} + \Delta E_{NPB}$) terms (Eq. (2)). (Right) Overall electrostatic contribution $\Delta G_{ele}$ as a function of the total positive charge carried by dendrimers 1–6. Filled and open symbols are used to distinguish between S=P core- and N3P3 core-based molecules. Data standard deviations are in the range $\pm 0.05$ to $\pm 0.15$.

Fig. 5. (Left) Subdivision of VPD molecules (Fig. 1) into chemically consistent fragments (SPC: S=P core; NPC: N3P3 core; MBH: 1-methyl-2-(4-methylbenzylidene)hydrazine fragment; BPC: inner 1,1'-diethyl-4,4'-bipyridine-1,1'-dium fragment; CCC: benzene fragment; BPF: external 1,1'-diethyl-4,4'-bipyridine-1,1'-dium fragment (only for dendrimers 5 and 6); BZA: benzaldehyde terminal group (only for dendrimers 1 and 2); DEP: -PO(OEt)2 terminal group (only for dendrimers 3 and 6)). (Right) Equilibrated molecular dynamics snapshots of dendrimers 1–6 in complex with HSA. Each protein residue involved in binding is labeled. The protein is in colored ribbon representation while the dendrimer molecules are shown as colored sticks-and-balls (1, golden rod; 2, orange red; 3, yellow; 4, firebrick; 5, gold; 6, red). Hydrogen atoms, water molecules, ions and counterions are not shown for clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
2.916, and 3.984 kcal/mol, respectively, whilst the SDGb,resHSA values for dendrimers 2, 4, and 6, characterized by the presence of the N3P3 core, are equal to 2.778, 5.564, and 4.972 kcal/mol, respectively (Tables SI4 and SI5). Interestingly, however, this “protein perspective” also confirms the less is more mantra. Indeed, especially for the dendrimers with Nc = 6, increasing dendrimer generation (and, hence, structural complexity) is detrimental to protein/dendrimer adaptation and, ultimately, effective binding.

The final result of this analysis is that dendrimer 4 represents the best compromise in terms of structure, charge optimization and terminal group; as such, it is predicted to be the most adaptive and effective HSA binder of the entire viologen-phosphorous dendrimer series considered.

3.3. Conformational changes of HSA upon binding viologen-phosphorous dendrimers 1–6

According to the in silico analysis of the binding thermodynamics of VPDs with human serum albumin reported above, all dendrimers 1–6 should be endowed with high affinity towards HSA. However, tight ligand/protein interactions may induce conformational changes in the polypeptide structure, which, in turn, may result in partial/substantial protein unfolding and loss of functionality/degradation. To check whether this was the case for the present systems, we further analyzed the molecular dynamics trajectories of each dendrimer/HSA complex and compared the corresponding secondary structure of the dendrimer-bound protein with that obtained for the free HSA in solution.

The analysis of the secondary structural motifs of the HSA alone and in complex with all VPDs 1–6 is reported in Table 3. As can be seen from this Table, dendrimer/HSA binding is accompanied by a negligible, if any, protein conformational change; this is particularly true for dendrimers 3–6, which all feature the -PO(OEt)2 terminal group. Structural changes in protein induced by ligand binding are crucial mechanisms of action and regulation/deregulation of protein biological activity. Accordingly, the preservation of HSA structural integrity upon binding of viologen-phosphorous dendrimers 1–6 under conditions of low dendrimer concentration is a very interesting prediction, which further supports the exploitation of HSA as an effective endogenous nanocarrier for the VDPs.

3.4. Experimental validation

3.4.1. Fluorescence quenching of HSA by viologen-phosphorous dendrimers 1–6

Fluorescence techniques provide several advantages compared with other biophysical and biochemical methods for measuring protein/ligand interactions. First, fluorescence intensity is linearly dependent on the number of fluorophores in a sample, providing a basis for quantitative measurements. Second, fluorescence measurements possess a very high sensitivity and thus can be performed on single molecules, providing the opportunity to observe biological mechanisms on a molecular level. Third, fluorescence is a process characterized by a range of different parameters, which can be measured independently or in combination providing information not only on the mere presence of a fluorophore but also on...
its orientation and its immediate environment.

HSA possesses only one single tryptophan residue (W214), located in the subdomain IIA. Accordingly, the fluorescence intensity behavior of HSA W214 in the presence of the different VPDs 1–6 is a suitable technique to study the underlying protein/dendrimer intermolecular interactions. As expected, the progressive addition of each dendrimer to the protein solution resulted in the regular decrease of W214 fluorescence intensity (as exemplified for dendrimer 4 in Figure S12), thereby confirming that all these viologen-based dendrimers were able to quench HSA.

Fluorescence quenching is usually classified as dynamic (DQ) or static (SQ) quenching. Generally, the two mechanisms can be distinguished by their different dependence on temperature. Higher temperatures result in faster diffusion and larger amounts of collision quenching, and will typically lead to the dissociation of weakly bound complexes and smaller amount of static quenching [64]. Accordingly, the value of the quenching constant \( k_q \) should increase for DQ while it should decrease for SQ with increasing temperature [65]. To determine the HSA quenching mechanism exerted by dendrimers 1–6, fluorescence-quenching data were analyzed using the well-known Stern–Volmer equation:

\[
F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q]
\]

in which \( F \) and \( F_0 \) are the fluorescence peak intensities of the fluorophore (i.e., W214 in HSA) in the absence and presence of quencher, respectively, \( k_q \) is the quenching rate constant of HSA, \( \tau_0 \) is the average life time of the protein (5.6 ns for HSA [66]), and \([Q]\) is the concentration of the quencher (dendrimers in the present case). The product of \( k_q \) and \( \tau_0 \) is also known as the Stern–Volmer quenching constant \( K_{SV} \). The Stern–Volmer plots for the quenching of HSA by dendrimers 1–6 at room temperature are shown in Fig. 7, together with the relevant values \( k_q \), while calculated \( K_{SV} \) are listed in the first column of Table 4.

As can be seen in Fig. 7, the values for \( k_q \) calculated using Eq. (5) are in the range \( 10^{12}–10^{13} \text{ M}^{-1} \text{ s}^{-1} \). Since all \( k_q \) values are much larger than the maximum collisional quenching constant \( (2.06 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}) \), SQ is the dominant quenching mechanism for all dendrimer/HSA complexes [67]. To further verify this assertion, the dependence of \( k_q \) on temperature was investigated. Accordingly, the Stern–Volmer plots for the quenching of HSA by dendrimers 1–6 at two further temperatures (i.e., 303, and 310 K) were determined (Table S16). The inverse correlation of \( k_q \) with temperature supports the evidence that the fluorescence quenching of HSA by all was initiated by complex formation between HSA dendrimers 1–6 rather than by dynamic collision between the two macromolecules [68,69]. The static nature of HSA fluorescence quenching by dendrimers 1–6 was obtained from time-resolved fluorescence analysis. Fig. 8 shows the fluorescence decay of HSA in the absence and presence of the viologen-phosphorous

---

**Table 4**

<table>
<thead>
<tr>
<th>Dendrimer</th>
<th>( K_{SV} ) (L/mol)</th>
<th>( K_q ) (L/mol)</th>
<th>( n )</th>
<th>( \Delta G_b ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( 1.84 \times 10^5 )</td>
<td>( 3.49 \times 10^6 )</td>
<td>1.057</td>
<td>-8.93</td>
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<tr>
<td>2</td>
<td>( 0.86 \times 10^6 )</td>
<td>( 9.30 \times 10^6 )</td>
<td>1.198</td>
<td>-9.51</td>
</tr>
<tr>
<td>3</td>
<td>( 3.73 \times 10^6 )</td>
<td>( 2.02 \times 10^7 )</td>
<td>1.203</td>
<td>-9.96</td>
</tr>
<tr>
<td>4</td>
<td>( 7.34 \times 10^6 )</td>
<td>( 8.85 \times 10^8 )</td>
<td>1.350</td>
<td>-13.59</td>
</tr>
<tr>
<td>5</td>
<td>( 4.08 \times 10^6 )</td>
<td>( 7.21 \times 10^7 )</td>
<td>1.113</td>
<td>-10.76</td>
</tr>
<tr>
<td>6</td>
<td>( 1.21 \times 10^7 )</td>
<td>( 2.45 \times 10^8 )</td>
<td>1.289</td>
<td>-11.45</td>
</tr>
</tbody>
</table>

---

**Fig. 7**. Stern–Volmer plots and \( k_q \) values for the quenching of HSA by dendrimers 1–6 at 298 K. [Dendrimer]: 1, 2, 3, 5, 7, 10, 12, 14, 16, 18, and 20 \( \times 10^{-6} \) M [HSA] = \( 5 \times 10^{-6} \) M.

**Fig. 8**. Time-resolved fluorescence decays of HSA \((1 \times 10^{-6} \text{ mol/L})\) alone and in the presence of the viologen-phosphorous dendrimer 6 \((5 \times 10^{-6} \text{ M})\).
dendrimer 6 as an example.

As it can be seen, HSA exhibits a single exponential decay in both cases; moreover, by increasing dendrimer concentration practically no change in HSA lifetime was observed (i.e., from 5.54 ns to 5.46 ns). Utterly analogous profiles and behaviors were observed for all other VPDs considered (data not shown).

For static quenching and under the assumption that there are $n$ equivalent and independent binding sites for the quencher ($Q$) on the protein, the binding constant $K_b$ for all viologen-phosphorous dendrimers 1–6 onto HSA can be calculated according to the modified Stern–Volmer equation:

$$\log([F_0 - F]/F) = \log K_b + n \log [Q] \quad (6)$$

where $F$ and $F_0$ denote steady-state fluorescence of HSA with and without quencher, respectively, $K_b$ is the binding constant, $n$ is the number of quencher molecules bound to HSA, and $[Q]$ is the concentration of the quencher (again dendrimers in the present case). The linear plots of $\log([F_0 - F]/F)$ vs. $\log [Q]$ obtained for all dendrimers 1–6 at 298 K are shown in Fig. 9, while the corresponding values of $K_b$ and $n$ are listed in the third and fourth columns of Table 4.

The same analysis was repeated for data obtained at 303 K and 310 K (Figure S13), and the corresponding $K_b$ and $n$ values for all dendrimers considered are gathered in Table 5.

It can be seen from Tables 4 and 5 that supramolecular complexes between HSA and dendrimers 1–6 can be easily formed at all temperatures; also, the number of binding sites $n$ is always close to 1, indicating that there is only one preferential dendrimer binding site on the protein surface. Moreover, the $K_b$ values presented in Tables 4 and 5 reveal that the fluorescence-derived experimental affinity of the present dendrimer family towards HSA follows the order: 4 > 6 > 5 > 3 > 2 > 1, in full agreement with computational predictions (see Table 2). In other words, dendrimer 4 is the strongest HSA binder ($\Delta G_b = -13.59$, $-13.14$, and $-12.81$ kcal/mol) whereas dendrimer 1 is endowed with the lowest protein binding capacity ($\Delta G_b = -8.93$, $-8.63$, and $-8.41$ kcal/mol) in the temperature range of physiological interest considered.

In the limited range of temperature values such as those considered in this work (i.e., $\Delta T = 12$ K), the variation of enthalpy with temperature can be neglected, so that the enthalpy value for the binding of dendrimers 1–6 to HSA can be calculated by applying the van’t Hoff equation:

$$\ln \left( \frac{K_{b,2}}{K_{b,1}} \right) = \frac{\Delta H_b}{R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \quad (7)$$

in which $R$ is the gas constant (1.987 cal/(mol K), $T_1$ and $T_2$ are two absolute temperature values (K), and $K_{b,1}$ and $K_{b,2}$ are the binding constant at $T_1$ and $T_2$, respectively.

Thus, choosing $T_1 = 298$ K and $T_2 = 310$ K, and inserting the corresponding values of $K_{b,1}$ and $K_{b,2}$ (Table 5) into Eq. (7), the relevant value of $\Delta H_b$ can be estimated. Also, using the corresponding $\Delta G_b$ values in Table 4, the entropic component $T\Delta S_b$ can be readily obtained from Eq. (8) as:

$$T\Delta S_b = \Delta H_b - \Delta G_b \quad (8)$$

Table 6 shows the results of this analysis. As it can be seen, the experimentally-derived enthalpic and the entropic component of the free energy of binding $\Delta G_b$ of dendrimers 1–6 onto HSA are negative at all temperatures, implying that the binding process between all dendrimers and the protein is predominantly enthalpic, while the entropic variation opposes binding, as most often seen for ligand/protein complexation and discussed in the relevant modeling section. Also, the opposite sign of $\Delta H_b$ and $T\Delta S_b$ confirm that electrostatic intermolecular interactions are

![Fig. 9. Modified Stern-Volmer plot for the quenching of HSA by viologen-phosphorous dendrimers 1–6 (panels A to F) at 25 °C. [HSA] = 5 \times 10^{-6} M.](image-url)
the number of binding sites (binding thermodynamics of viologen-phosphorous dendrimers). Accordingly, ITC experiments were performed to determine the endothermic phenomena observed in the ITC experiments. In particular, it action, and the type of interactions depending on the exothermic or associated or a disassociation process, the binding af

\[ \Delta G_b = \Delta H_b - T \Delta S_b \]


dendrimer

\[ G_b \]

was obtained via Eqs. (7) and (8), using the \( K_b \) and \( \Delta G_b \) values reported in Table 5 (see text for details).

Table 6

<table>
<thead>
<tr>
<th>Dendrimer</th>
<th>( T (K) )</th>
<th>( \Delta H_b ) (kcal/mol)</th>
<th>( -\Delta S_b ) (kcal/mol)</th>
<th>( \Delta G_b ) (kcal/mol)</th>
</tr>
</thead>
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<td>1</td>
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<td>+4.59</td>
<td>-8.93</td>
</tr>
<tr>
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<td>303</td>
<td>-8.63</td>
<td>-5.11</td>
<td>-8.41</td>
</tr>
<tr>
<td>310</td>
<td>310</td>
<td>-9.51</td>
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<td>2</td>
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<tr>
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<td>-9.63</td>
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</tr>
<tr>
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<td>310</td>
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<td>-4.52</td>
</tr>
<tr>
<td>4</td>
<td>303</td>
<td>-13.59</td>
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<td>-7.39</td>
</tr>
<tr>
<td>310</td>
<td>303</td>
<td>-13.14</td>
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<td>-6.59</td>
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<td>5</td>
<td>310</td>
<td>-12.81</td>
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<td>310</td>
<td>-10.81</td>
<td>+5.64</td>
<td>-6.17</td>
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</tbody>
</table>

prevalently leading the HSA/dendrimer binding process as anticipated by modeling results and expected given the nature of the receptor and its ligands.

3.4.2. Thermodynamics of binding of HSA and viologen-phosphorous dendrimers 1–6 via isothermal titration calorimetry (ITC)

ITC is a highly sensitive technique, which measures the enthalpy change of a reacting system as a function of reactant amount. It is recognized as one of the most popular techniques used for investigating intermolecular and/or intramolecular interactions during the past decade [70]. It simultaneously gives the enthalpy change (\( \Delta H_b \)), entropy change (\( \Delta S_b \)), and Gibbs energy change (\( \Delta G_b \)) of an association or a dissociation process, the binding affinity (\( K_b \)) and the number of binding sites (\( n \)) of the receptor-ligand binding reaction, and the type of interactions depending on the exothermic or endothermic phenomena observed in the ITC experiments. Accordingly, ITC experiments were performed to determine the binding thermodynamics of viologen-phosphorous dendrimers 1–6 to human serum albumin at room temperature. A representative ITC curve is shown in Fig. 10 for the binding of dendrimer 6 with HSA, whereas the full set of ITC results is listed in Table 7.

In agreement with simulation predictions and fluorescence spectroscopy evidences, ITC data yield values of the binding constant \( K_b \) for all VPDs onto HSA in the range \( 10^5–10^9 \) L/mol, so confirming that the binding affinity of all dendrimers for this protein is very high. As verified by ITC, the values of the parameter \( n \) are also close to 1 for all VPD/HSA complexes (Table 7, second column), thereby validating the underlying computational assumption of a 1:1 stoichiometry for each protein/dendrimer assembly formation. Most importantly, however, the ITC estimated values of the binding free energy \( \Delta G_b \) and its enthalpic and entropic contributions (\( \Delta H_b \) and \( -\Delta S_b \), respectively) not only show the same qualitative trend but are also in excellent quantitative agreement with those derived from the application of the MM/PBSA computational ansatz, as can be inferred from a comparison of the corresponding values listed in Tables 4 and 7. The first three panels of Fig. S14 give a graphical portrait of this nice correlation. This allows us to conclude that the MM-PBSA method is reliably reproducing the free energy of binding of the six viologen-phosphorous dendrimers 1–6 with human serum albumin and performs remarkably well for rank-ordering the differential affinity of each VPD towards this protein.

3.4.3. Circular dichroism (CD) spectroscopy analysis

Circular dichroism (CD) spectroscopy is a widely used method to determine protein conformation, structure and stability in solution, and to monitor interactions between proteins and other molecules. Accordingly, CD spectra of HSA alone and in the presence of increasing concentrations of dendrimers 1–6 were recorded to decipher the structural and conformational changes, if any, exerted by dendrimer binding on the secondary structure of the protein. Fig. 11 shows the results obtained for the system HSA/A4 as a proof-of-concept. As seen in this Figure, the CD spectrum of native albumin exhibits two negative absorption bands in the far-UV region, peaking at 208 nm (\( \pi-\pi^* \)) and 222 nm (\( n-\pi^* \)), characteristic of the \( \alpha \)-helix portions of the protein [71]. The addition of dendrimer 4 to the HSA solution results in minimal reduction in band intensity at all wavelengths of the relevant CD spectra, without any discernible shift of the band maxima. This increase in ellipticity suggests a stabilization of the complex with respect to free HSA. In other words, the intermolecular interactions between the dendrimers and HSA do not substantially perturb the \( \alpha \)-helical motifs of the protein to an extent to leave a signature on the relevant CD spectra. This, in turn, implies that the dendrimers molecules cannot penetrate inside the structure of HSA but, rather, they bind to the protein surface, in agreement with the molecular modeling predictions discussed in paragraphs 3.2 and 3.3. This somewhat expected result could be further rationalized considering that HSA is a single polypeptide chain characterized by the presence of 17 disulfide bridges, located at regular intervals along the protein structure. Accordingly, in addition to its general compact folded nature, HSA flexibility is considerably restricted by virtue of this network of 5–5 links. Hence, when dendrimer 4 interacts with HSA in solution, the protein native structure is not expected to unfold to a considerable extent due to 4 surface binding.

Table 8 shows the analysis of the secondary structural motifs of the HSA per se and in complex with VPDs 1–6 as extracted from the corresponding CD data obtained at the lowest dendrimer/HSA molar ratio (see Table S17 for data at all molar ratios). From this table it is clear that all six dendrimers do not induce any significant conformational change in the protein for all values of the dendrimer/HSA molar ratio (MR). Pleasingly, this result agrees with the HSA secondary structure analysis predicted by computer

![Fig. 10. (Inset: ITC raw data for the interaction between the viologen-phosphorous dendrimer 4 and HSA at room temperature in PBS buffer at pH = 7.4.) Plot of the integrated area under each ITC peak for the same system. The solid line is model data fitting with adjustable parameters n, K_b, and ΔH_b. (see text for details).](image-url)
Table 7
Thermodynamic parameters for the binding of dendrimers 1–6 to HSA at 298 K (see text for details).

<table>
<thead>
<tr>
<th>Dendrimer</th>
<th>n</th>
<th>$K_b$(L/mol)</th>
<th>$\Delta H_b$(kcal/mol)</th>
<th>$-\Delta S_b$(kcal/mol)</th>
<th>$\Delta G_b$(kcal/mol)</th>
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<tr>
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Fig. 11. Example of far UV-CD spectra of HSA in the presence of increasing concentrations of VPD 4 at room temperature. MRE = mean residue ellipticity. 4/HSA molar ratio from a to e: 0, 1, 5, 10, and 20.

Table 8
Composition of the secondary structure of HSA alone and in complex with VPDs 1–6 at dendrimer/HSA molar ratio = 1 as determined from CD measurements.

<table>
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<tr>
<th>HSA alone</th>
<th>% $\alpha$-helix</th>
<th>% Antiparallel</th>
<th>% Parallel</th>
<th>% Turn</th>
<th>% Random coil</th>
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<table>
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<th>% turn</th>
<th>% random coil</th>
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4. Conclusions

Dendrimers constitute a class of perfectly defined, self-similar macromolecules, whose architecture consists in a central core, from which identical branches emanate radially, and comprise identical layers, termed generations. One of the most interesting aspects of dendrimers resides in the so-called dendritic (or dendritic) effect [9], which is observed when a specific chemical function exhibit different properties, depending on whether it is grafted or not to a dendrimer scaffold. Specifically, if all generations of a given dendrimer are endowed with the same properties but the monomer does behave differently, then this effect is defined as multivalency. On the other hand, if the considered property varies with dendrimer generation, the phenomenon is called generation effect, the underlying consequence being most often ascribable to the dendrimer terminal groups.

Dendrimers featuring 4,4'-bipyridinium salts (i.e., viologens) as subunits have recently become very attractive due to the peculiar properties of this chemical moiety. In fact, viologens are well-known electrochromic materials, and can form strong charge-transfer complexes with a variety of suitable donor compounds [72]. Thus, a plethora of dendrimers bearing the viologen group at the core, along the branches, or at their periphery have been synthesized, characterized, and substantial efforts have been devoted to the optimization of the relevant synthetic strategies, specific functionalization, and physical and biological applications [73–75].

Phosphorous is an element that plays a key role in almost all aspects of life. Thus, in the form of the triple-negatively charge phosphate anion it is a fundamental constituent of nucleic acids, phosphorylation is the main starting event in the cast majority of all cellular signaling cascades, mixtures of phospholipids constitute cellular membranes and, last but not least, inorganic phosphates are vital components of bones. It should not be surprising then that chemicals containing phosphorous in their formula may interfere (either positively or negatively) with biological systems. Accordingly, the idea of incorporating phosphorous into the chemical structure of dendrimers resulted in phosphorous-containing dendrimers, which were proven to exhibit a variety of roles when in contact with biomolecular entities [13].

Human serum albumin constitutes some 50% of the protein present in the plasma of normal healthy individuals. Albumin is the main determinant of plasma oncotic pressure and it plays a pivotal role in modulating the distribution of fluids between compartments. Moreover, it has many other biological properties which may be important not only for its physiological actions but also for its therapeutic effects. Among these are its capacity of molecule transportation and free radical scavenging, its ability to modulate capillary permeability, neutrophil adhesion and activation, and its hemostatic effects [1].

Notwithstanding the potential perspective of biomedical applications of viologen-phosphorus dendrimers outlined above, unexpectedly no studies beyond basic biological properties of VPDs have been published so far. Therefore, with the aim of exploiting HSA as a potential carrier for VPDs, in this work we performed a preliminary investigation of the interaction of the six different VPDs 1–6 with HSA using a combination of computational and experimental techniques. Accordingly, different simulations...
methodologies were employed to determine the dendrimer/HSA binding mode and the relevant binding thermodynamic parameters. All molecular determinants contributing to VPDS/HSA free energy of binding ∆Gb were analyzed, and the eventual HSA structural variations induced by dendrimer binding were quantified. Finally, all modeling predictions were validated using a series of experimental approaches, including isothermal titration calorimetry (ITC), circular dichroism (CD), and fluorescence quenching and decay (see also last Panel in Figure S1A). In summary, the results from this study allowed us to rank the affinity of the different viologen-phosphorous dendrimers 1–6 towards HSA and to formulate a molecular-based rationale for the differential binding thermodynamics of the resulting dendrimer/HSA complexes. According to our data, HSA can successfully and selectively bind VPDS 1–6, dendrimer 4 being the best cargo for this endogenous protein nanocarrier.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fluid.2016.02.014.

References
