# MOLECULAR DOCKING STUDY ON THE POTENCY OF GLYCOSAMINOGLYCANS (GAGs) AS CO-ACTIVATORS OF FIBROBLASTS PROLIFERATION AND DIFFERENTIATION

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**Abstract:** Fibroblast growth factors (FGF) are heparin-binding polypeptides that control differentiation, growth and migration of many cell types. The signaling in the FGF pathway implies the dimerization of their corresponding cell surface receptors and consequently their activation through autophosphorylation of their cytoplasmatic domains. This process is modulated by heparan-sulfates (HS) or heparan-sufate-proteoglycans (HSPG) present in great abundance on cell surfaces. Different aspects of this modulation were a matter of debate in the last years in the field literature and some of them still remains unclear. The present study is trying to give some answers to a part of these problems by using quantitatively physical theoretical models of the molecules involved in these processes. We aimed to explain, through the aid of molecular docking techniques, the experimental findings about the activation of FGF receptors by small saccharides (di- and tetrasaccharides) and as a result we propose a model for this process. Also, the findings resulted from docking experiments of longer oligosaccharides (hexa- and octasaccharides) offer insights about the stoichiometry of the receptorial complex, supporting a **(2:2:2)**FGF:FGFR:HS ratio scheme rather than a **(2:2:1)**FGF:FGFR:HS one, in accordance with recent experiments on the subject published in the literature.

## INTRODUCTION

Growth factors are chemical substances involved in inter- and intracellular signaling that act to produce a series of reactions including cellular migration and adhesion, collagen synthesis, metalloproteinase production for the extracellular matrix, and integrins expression. Tissue and cellular diversity as well as specificity of the FGF signaling result from the combinatorial oligomerization of the fibroblast growth factor family, to different ectodomains, derived from the expression of the various segments of the four codifying genes of the membrane receptor tyrosine-kinases; the oligomerization is modulated by oligosaccharides contained in the chains of heparan-sulfate (HS) and heparan-sulfate-proteoglycans.

Growth factor receptors belong to the receptor tyrosine kinases family (RTK), which are able to catalyse the transfer of the γ-phosphate from the ATP to the hydroxyl groups of the tyrosine residues on the intracellular target proteins. The RTK family has numerous members, but they all have in common the fact that they are activated through dimerization. At present there are four known types of receptors for the fibroblast growth factors ($FGFR_{1-4}$). The alternative splicing of the exons which codify the extracellular domain of the FGFR1-3 receptors leads to the “c” and “b” isoforms, that are characterized by unique binding features and specific tissue locations (Yeh, 2003). The transmembrane domain of the receptor consists of a single α-helix that traverses the membrane entirely. The cytoplasmatic domain consists of a conserved protein-kinase fragment and additional regulating sequences that are prone to autophosphorylation and phosphorylation under the action of some heterologue tyrosin kinase proteins.

So far the crystallographically determined structures of two types of FGF-FGFR dimerized complexes are known, that is those with the registration codes 1E0O (PDB) for a **(2:2:1)**$FGF_2$:$FGFR_1$:HS ensemble (Pellegrini, 2000) and respectively 1FQ9 in the Protein Data Bank (PDB) for a **(2:2:2)**$FGF_2$:$FGFR_1$:HS ensemble (Schlessinger, 2000). These models sustain the two imagined mechanisms for the dimerization of the FGFR receptors. The first mechanism (antisymmetric model) of receptor dimerization implies the interaction of the two $FGF_1$:$FGFR_2$ complexes with a single HS molecule. The two $FGF_1$ ligands only come into contact with a single $FGFR_2$ receptor, and the HS molecule positively influences the affinity that $FGF_1$ has for $FGFR_2$ only for one of the monomeric $FGF_1$:$FGFR_2$ complexes. The stoichiometry of the activated receptor system is in this case **(2:2:1)**$FGF_1$:$FGFR_2$:HS.

The second model (symmetric two-end model) proposes the receptor oligomerization as a symmetrical dimeric ensemble of $FGF_2$:$FGFR_1$:HS monomers where the $FGF_2$ ligands simultaneously interact with both $FGFR_1$ receptors. The affinity of $FGF_2$ for the $FGFR_1$ is amplified by the HS molecules that bind on specific sites on both molecules. HS molecule on one of the $FGF_2$:$FGFR_1$ monomers has at the same time the role of encouraging the dimerization of the $FGF_2$:$FGFR_1$:HS complexes through the supplementary interaction with the $FGFR_1$ receptor belonging to the other identical $FGF_2$:$FGFR_1$:HS monomer. The stoichiometry of the activated receptor system is thus **(2:2:2)**$FGF_2$:$FGFR_1$:HS. Recent studies based on the structural crystallographic analysis, biochemical methods, mass spectroscopy (MALDI-TOF), and mutagenesis (Ibrahimi, 2005) considers the symmetric two-end model as the most probable one. But even if this is

the case the stoichiometry is not clear because there is still the possibility that a single molecule of HS can bind transversely along the receptorial complex and interact both with the two receptors and with the two ligands at the same time in which case the stoichiometry is **(2:2:1)**$FGF_1$:$FGFR_2$:HS. Having these as a motivation, our attention was focused on the 1FQ9 structure as the main candidate for the docking studies presented below. The method of molecular modeling is a very appealing choice in this respect because the phenomena studied manifests itself at molecular spatial and temporal levels and thus it can offer insights into the very intimate mechanisms displayed in FGF signaling.

Having these in mind, the idea of extensive docking research of some representatives of the HS class with oligomeric ensembles of FGF:FGFR took shape. The present study is concerned with the analysis using molecular modeling of the way that heparan-sulfates (heparin, HSPG) are involved in the receptor activation by oligomerization with the fibroblast growth factors. The results are finally discussed as seen through the models of activating FGFR and compared with the mechanisms from the literature, imagined subsequently to experimental studies, especially crystallographic.

As an addition, here we made an attempt to create a model for FGFR activation in the presence of small dimension HS – disaccharides, tetrasaccharides and hexasaccharides – subject to controversy as for their ability to trigger the oligomerization of the FGFR receptors that is the starting point of transducing the FGF signal.

Hence there is data that confirms the co-activation of the FGFR receptors by disaccharides and hexasaccharides. Nevertheless, the lack of crystallographically determined structures that would reveal the actual way of binding of small HS to establish these FGF:FGFR:HS oligomeric complexes leads to the absence of the necessary theoretical basis to more in depth understanding of this process. This absent "niche" in experimental data can be partially filled with docking studies using algorithms and forcefields previously validated and accepted in molecular docking research.

## MATERIALS AND METHOD

The methodology chosen for modeling consisted of simulating the docking of HS molecules with one, two, three, and respectively four disaccharidic units on an oligomeric **(2:2)**$FGF_2$:$FGFR_1$ complex. The molecular docking algorithms generally follow the prediction of conformation and contact areas between two interacting molecules, such as the protein-ligand and protein-protein complexes. The topic of predicting this interaction is known in the field literature as the *docking problem*. Specifically, in the case of two biological molecules with a known tridimensional structure, solving the docking problem should answer the following question: what is the conformation of the ensemble formed by these two molecules when they interact? The molecules can be proteins, nucleic acids, poly- and oligo-saccharides or small molecules such as substrates and inhibitors. The docking algorithm identifies conformations that are most energetically favorable to the ligands, in the form of the lowest *binding free energies*. Energy is most often evaluated using empirical forcefields that contain several terms usually associated with the conformational degrees of freedom, van der Waals and electrostatic interactions. The generally accepted principle considers that conformations with the lowest free energy of binding best reflect the real way of interacting between a ligand and a macromolecule. The docking problem thus comes down to an optimization problem where the task is finding the energetically solutions with the smallest value. Widely used classes of algorithms at present for automated docking are the Evolutionary Algorithms (EA). Evolutionary algorithms are a group of methods based on the principle of biological evolution that are such designed to solve optimization problems. There are three main classes of EA: Genetic Algorithms (GA), Evolutionary Programming (EP), and Evolution Strategies (ES) (Leach, 2001).

In this study we have chosen to use an "in lab" modified (to accept more active torsions) version of the AutoDock 3.05(Morris, 1998) program because it does not require previous knowledge of the binding site of a protein and because of its speed and high accuracy. Although information offered by the crystallographic structures for the HS:FGF, HS:FGFR, FGF:FGFR:HS complexes identify these sites for HS including respectively 3, 4, 5 disaccharidic units, "Blind Docking" was preferred, meaning the docking attempt without previous knowledge of the binding site, for two reasons: firstly to validate the method used we tried to reproduce by computation the crystalographically observed complexes for heparan-sulphates of 3 and 4 disaccharidic units, and secondly because there is no exact (experimental) knowledge about the binding modes of heparan-sulphates of small dimensions (one or two disaccharidic units) in a **(2:2)**FGF:FGFR complex.

The structure the dockings of heparan-sulfates have been made on was the one with the registration code 1FQ9 in the Protein Data Bank. The strucure of heparan-sulfates was generated using the program Arguslab ver.4.0 (Planaria Software LLC, 2004: http://www.planaria-software.com/) as follows:

- **HS2 (disaccharide): IdoA** – ($\alpha$1-4) – **GlcN**
- **HS4 (tetrasaccharide): IdoA** – ($\alpha$1-4) – **GlcN** – ($\beta$1-4) – **IdoA** – ($\alpha$1-4) – **GlcN**
- **HS6 (hexasaccharide): IdoA** – ($\alpha$1-4) – **GlcN** – ($\beta$1-4) – **IdoA** – ($\alpha$1-4) – **GlcN** – ($\beta$1-4) – **IdoA** – ($\alpha$1-4) – **GlcN**
- **HS8 (octasaccharide): IdoA** – ($\alpha$1-4) – **GlcN** – ($\beta$1-4) – **IdoA** – ($\alpha$1-4) – **GlcN** – ($\beta$1-4) – **IdoA** – ($\alpha$1-4) – **GlcN**– ($\beta$1-4) – **IdoA** – ($\alpha$1-4) – **GlcN**

Two sets of docking simulations have been performed:

1. In the first set all torsion angles in the HS molecules were considere flexible (active)

2. In the second set the torsion angles of HS6 and HS8 corresponding to the glycosidic linkages were considered inflexible (inactive) and fixed at a value corresponding to the helical structure of heparin/HS in a water solution (Mulloy, 1993).

All simulations have been run in the Linux RedHat 9.0 operating system, on two computers having Pentium IV- 3.0 GHz processors, 1Gb RAM. The total amount of time for all simulations was about 700 hours of continuous calculation.

## RESULTS AND DISCUSSIONS

In the first stage we followed the reproduction through docking simulations of the binding manner of HS that was observed experimentally in the crystallographic structure 1FQ9 on the **(2:2)**$FGF_2$:$FGFR_1$ complex, to verify and validate the simulation algorithm. Thus, two sets of simulations were performed, where two molecules were bound on the above mentioned structure: one molecule of HS6 and one of HS8, using consecutively conformations described and the algorithms presented in the section “Material and Method”. The results are different in the case where no restrictions are imposed on the glycosidic torsion angles, as opposed to the case where they are considered constant and having a determined value according to the secondary structure of heparin in water **(Figure 1)**.

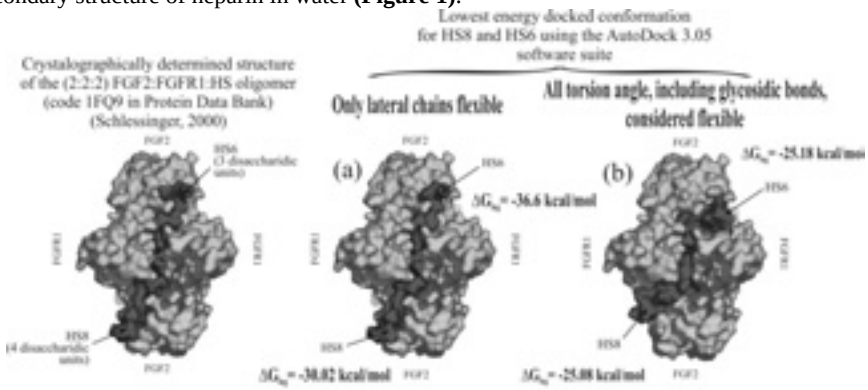


**Figure 1.** Molecular docking results for HS8 (4 disaccharidic units) and HS6 (3 disaccharidic units) on the (2:2) FGF2-FGFR1 complex. On the left side there is depicted the experimental determined structure of the dimerized complex in the presence of heparin sulfates and on nthe right the structures obtained in docking simulations on the same molecular components

We have found that the docking algorithm used is capable to track down the binding sites of heparan sulfates in both cases, but the runs in which the glycosidic bonds were considered active (flexible) fail to reproduce the exact conformation found in the experimental structure. This is probably related to the intimate molecular mechanism used by heparan sulfates to fulfill their biological function. As extensive attempts (600 structures for HS8 and 750 for HS6) to dock completely flexible HS on the FGF-FGFR complexes have failed, while imposing a well determined secondary structure (similar to that of heparin in water) leads to the success of the docking algorithm only after a small number of attempts (200 for HS8 and 500 for HS6), we are inclined to assume, as previously suggested by the experimental studies (Loo, 2001), that a well determined secondary structure of HS is a necessary requirement (but not necessarily sufficient) to induce FGFR dimerization by HS in cooperation with the FGF factors.

One important issue is related to the values of binding free energy obtained. It is clear that the values in the range of -30 ÷ -20 kcal/mol are too big to correspond to real experimental values. Ususlly, the noncovalent ligand binding processes on proteins are characterized by free energy in the range -2 to -15 kcal/mol (Trott, 2009). Our explanation for the results obtained in the case of heparin-sulfates is as follows. Docking programs generally use a scoring function, which can be seen as an attempt to approximate the standard chemical potentials of the system. When the superficially physics-based terms like the 6–12 van der Waals interactions and Coulomb energies are used in the scoring function, they need to be significantly

empirically weighted, to account on one hand for the difference between *energies* and *free energies* (the binding problem is governed not only by the energy profile but also by the shape of the profile and the temperature) and on other hand for the loss in conformational entropy in the process of binding. A drug-like molecule that binds a protein becomes less mobile, and the resulting loss in configurational entropy opposes the attractive forces that drive binding. A number of empirical energy models used in virtual ligand screening include a term to account for this entropic penalty (Chang, 2007). The Autodock 3.05 does not include such a term explicitly as its primary use is to predict dockings of small molecules (few torsional degrees of freedom) on rigid proteins. Instead it uses a set of compounds with experimentally known dissociation/inhibition constants for training the scoring function. Taking into account the fact that the oligosaccharide molecules used in the docking experiments presented here have a high number of flexible bonds there should be added a positive correction to the Autodock normal score function, which should draw the predicted free energy of binding to more positive values. On the other hand the molecules used as Autodock 3.05 score function training set are mostly not very electropositive or electronegative ones. In contrast our studied molecules are among the most electronegative charged natural compounds. This also contributes to a high affinity for the electropositive regions (binding sites) on the surface of the FGF receptors.

Taking into consideration the results presented in **Figure 1** and those previously stated, we consider that the method and algorithm used are valid for the structural study of binding HS that contains 1, 2, 3, and respectively 4 disaccharidic units (HS2, HS4, HS6, HS8) on FGF-FGFR oligomeric complexes, with the mention that the free energies predicted should be regarded qualitatively and just for comparison between different docked molecules from the same class.

One of the important issues brought into discussion along time, concerning the manner in which HS is involved in FGFR dimerization is the optimal length of the polisaccharidic chain that can trigger this dimerization. The analysis of the experimentally determined structures and the studies based on manual docking of HS on models of dimerized receptors lead to the conclusion that a maximally active dodecasaccharide chain bound in the central area of the electropositive canyon delimitated by the FGFR receptors and FGF ligands will interact with both the receptors and the FGF ligands. Such a molecule can in these circumstances cross all the space between the two receptors providing their cohesion while being capable at the same time to interact with the binding sites of heparin on both FGF factors. In this case the stoichiometry of the receptorial complex will be **(2:2:1)**FGF:FGFR:HS.

In contrast to that, an octasaccharide placed in the center of the space between the two receptors can completely interact only with the receptors, while interacting with the ligands in a minmal manner. A hexasaccharide molecule centrally placed in the canyon between the receptors can no longer provide the stability of the receptor complex because it no longer simultaneously interacts with both receptors and the FGF ligands.

As a result of these findings, it has been postulated that the HS with the minimal dimension that could induce dimerization and would thus be biologically active should be octasaccharidic (Ornitz, 1992). However, there still are studies proving that hexasaccharides (Gambarini, 1993; Zhou, 1997) and even disaccharides (Ornitz, 1995) have a biological activity in this respect. The structure crystalograpically determined by Schlessinger (2000) (code 1FQ9 in PDB) settles this contradiction by offering a direct image of the manner in which two HS molecules with a length of at least 6 saccharides can stabilize the **(2:2)**FGF:FGFR oligomeric complex. Thus it appears that the degree of polymerization of the polysaccharidic substance is not as important as is the *presence* of this substance in *well defined* areas on the interface between the growth factors (ligands) and the corresponding receptors. This idea is further enhanced by the finding that in the absence of heparin molecules a series of sulfate ions bind into the electropositive canyon between receptors and have been proposed to mimic the sulfate moieties of heparin (Plotnikov, 1999).

Besides, the manner of docking of long chain HS (3 or 4 disaccharidic units) can bring additional clues as for the controversies concerning the stoichiometry of the receptorial oligomeric ensemble.

The results of the HS2, HS4 docking on the **(2:2)**$FGF_2$:$FGFR_1$ structure are presented in **Figure 2** and **Figure 3** where there are presented the structures with the lowest binding energy, interesting when interpreting the way this class of compounds favor dimerization.

The small saccharidic molecules – HS2 and HS4 – are noticed to bind primarily on the same binding sites on the FGF factors and the FGFR receptors as the larger molecules of the HS6 and HS8 co-factors.

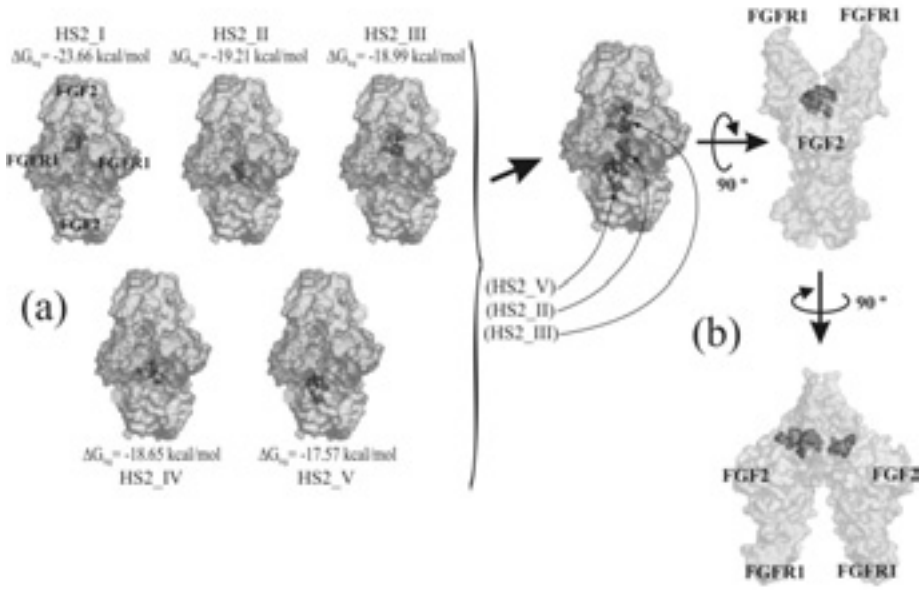


**Figure 2.** The five (HS2_I, HS2_II, HS2_III, HS2_IV) docked conformations with the lowest binding free energy of the HS2 (one disaccharidic unit).

Subsequent to the analysis of these binding manners, we propose the following dimerization model induced by the HS2 and HS4 oligosaccharides. The activating mechanism of FGFR receptors could be based on cooperative phenomena manifested by the small saccharidic molecules. A certain number of such molecules could create connecting bridges between the protein molecules of the receptors and those of the growth factors (see **Figure 2 and Figure 3**). The oligosaccharidic molecules interact through hydrogen bridges with the sidechains of the polypeptide chains of FGF and FGFR and can stabilize the **(2:2)**FGF:FGFR oligomeric complex in the following ways, noticed during the dockings:

1. by increasing the affinity of FGF factors for the FGFR as a result of the binding of saccharidic molecules on both FGF and the corresponding FGFR receptor.
2. by direct binding of the two FGFR1 receptors, the saccharidic molecule being placed in the electropositive area between them.
3. by increasing the affinity of the FGF factor in one monomeric complex, for the FGFR receptor in the other complex.
4. for the HS4 molecules there is also the possibility of ternary FGFR-FGF-FGFR interaction through a oligosaccharidic molecule that simultaneously interacts with both FGFR receptors and one of the growth factors.

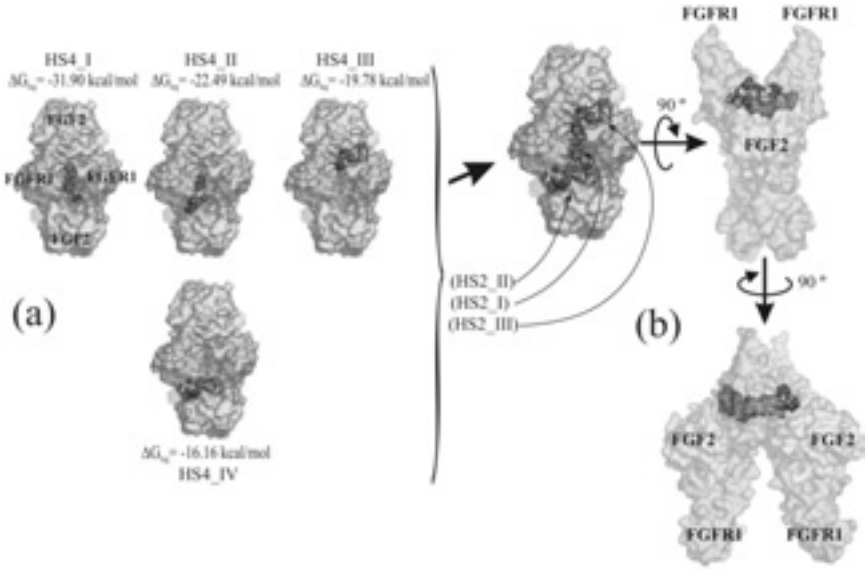


**Figure 3.** The five (HS4_I, HS4_II, HS4_III, HS4_IV) docked conformations with the lowest binding free energy of the HS4 (one disaccharidic unit).

In the **Figure 4** there are represented the results of the statistic analysis of the populations of structures obtained throughout the docking process. It is to be noticed that HS binding is accomplished in well defined areas on the molecular surfaces of the receptorial oligomer, such identified as binding sites of HS co-factors within the oligomer and which coincide with the binding sites of heparin/HS/HSPG proposed in the field literature as being active. At the same time, the distribution of the molecular geometrical centers of HS with chains larger than 6 saccharidic units implies the absence of docking of such a molecule in a central position in the space between the two receptors. From a total number of 200 docked conformations obtained in this case, not even one was found to be placed at the center of the receptorial complex. If we could obtained docked conformations at this position this would have meant that a single heparin/HS/HSPG molecule with at least 8 saccharides could have been capable to induce dimerization by simultaneous binding both with ligands and receptors, resulting in an oligomer with a **(2:2:1)**FGF:FGFR:HS stoichiometry.

The most recent studies on the other hand (based on crystallographic as well as biochemical results (Ibrahimi, 2005)) bring evidence that shows a **(2:2:2)**FGF:FGFR:HS stoichiometry for the dimeric receptorial state.

Another interesting research regarding the kinetics of the mechanism of FGRF activation shows, with the aid of Surface Plasmon Resonance technique, that the dimerization is promoted by firstly coupling between the FGF and HSPG, resulting in a complex with a high affinity for FGFR. After this first binding takes place the FGF:HSPG complex binds to FGFR (Ibrahimi, 2004). This stepwise assembly also sustain a **(2:2:2)**FGF:FGFR:HS stoichiometry. Therefore, the results of the docking study presented above can be regarded as a possible explanation for the observed stoichiometry of dimerization, pointing out the energetic or steric inadequacy of binding a single molecule of HS that should cover the entire receptorial complex.

It has to be stated however that there is also the possibility that the absence of protein side chain flexibility representation in the docking algorithm used may be the cause for the failure of docking a long (HS8) molecule in the central space between the receptors. One of the current problems with the present docking algorithms is the accurate inclusion of both protein side chain flexibility and a certain degree of backbone mobility for the target protein on which the docking takes place.

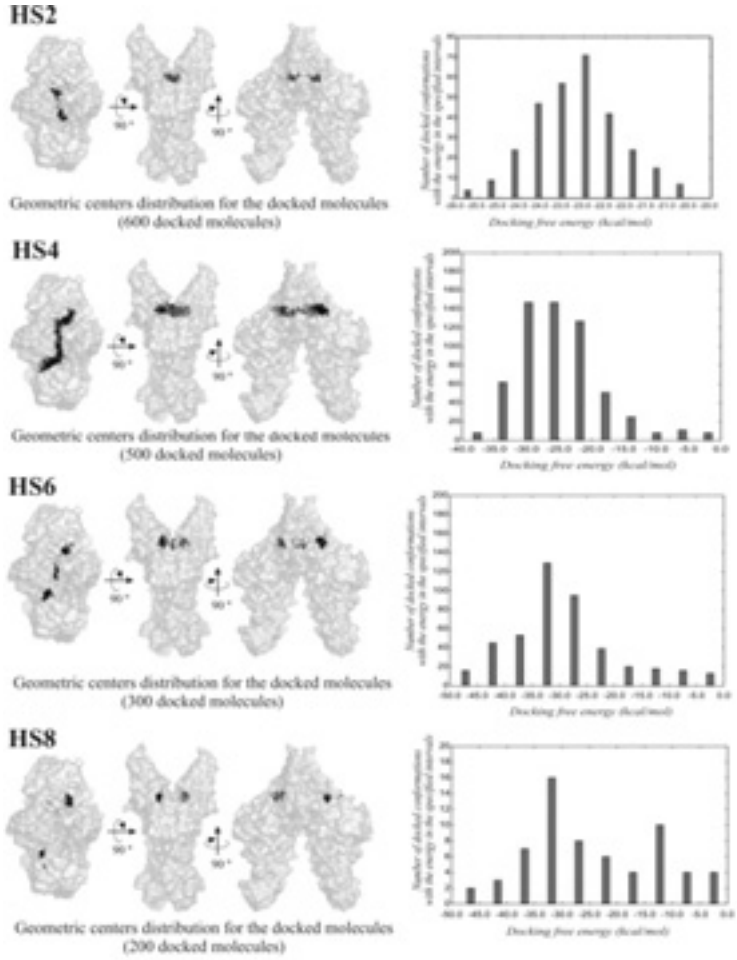


**Figure 4.** Statistical analysis of all the conformations resulted from docking simulations for HS2, HS4, HS6 and HS8

The findings presented here must be further investigated and reproduced with other software packages and with the forthcoming version of AutoDock (v. 4.0) which will have built in protein side chain flexibility capabilities.

## CONCLUSIONS

In the present study we have attempted to quantitatively approach aspects of the manner in which polisaccharidic co-factors (heparin/HS/HSPG) are involved in modulating the transmitting of information by signaling through fibroblast growth factors, using molecular modeling methods, already well implemented in the larger field of molecular biology and also in the course of an accelerated development due to the increasing capacity of present day calculation systems.

Emphasis should be placed on the fact that the system chosen for this study, that is the receptorial dimer consisting of two $FGFR_1$ receptors and two $FGF_2$ ligands can be seen as a general model for the class of receptors and of fibroblast growth factors, due to the high identity of the primary sequences and of the tridimensional structure that the receptors and the growth factors in this class manifest among themselves.

As a consequence of the study we propose an inducing mechanism of dimerization by the oligosaccharidic molecules of small dimensions di- and tetrasaccharides, their biological activity being proven by experimental studies. The mechanism is based on cooperative phenomena manifested by a population of such molecules that are capable to simultaneously bind to protein molecules and favor dimerization.

At the same time, the docking simulations of oligosaccharides with 6, and respectively 8 saccharid units have demonstrated that polysaccharidic chains of such or larger dimensions must be characterized by well defined secondary structures to be able to successfully bind to the elements constituting the receptorial oligomeric complex.

The total absence of certain HS8 molecules docked in the space between the two receptors deems as energetically or sterically unfavorable the situation in which a single HS molecule should cover the entire receptorial complex. An explanation is thus proposed for the experimentally found facts that suggests a stoichiometry of **(2:2:2)**FGF:FGFR:HS for the receptorial oligomer as the most probable one.

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