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Hybrid Polymeric Systems for Nano-Selective Counter Intervention in Virus Life Cycle

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Summary: Self assembly of viral biopolymers to nano-complexes forming virions during virus delivery from infected cell and reverse disintegration to virus entry into new cells play a crucial role in viral life cycle and in viral diseases. Therefore artificial instruments for selective counter intervention into these processes are dramatically required for the high effective antiviral protection. Hybrid macromolecular systems (HMS) rationally integrating heterogeneous structure-functional factors for selective recognition - inhibition of viruses (nano-objects) without detriment for cells (micro-objects) can become a molecular basis for cardinal progress in this area. Here we discuss approaches to design and current experimental results of synthesis, and antiviral selectivity evaluations of the HMS, based on combinations of polyelectrolyte-grafted components constructed on principles of mimicry and/or complementarity to viral targets or virus-sensitive cell receptors. Particularly, the HMS generations strongly inhibiting the human immunodeficiency virus (HIV) were created as platform to novel drug development against HIV/AIDS and other sexually transmitted infections.

Keywords: antiviral selective polymers; nanocomplexes; viruses

A Viral Life Cycle in the Focus of Nano-Scale Inter-Bio-Polymeric Complexes as Targets for Therapeutic Intervention

The typical viral life cycle (Fig. 1) can be considered as a replicable evolution of viruses from nano-scale objects and events (1. extracellular virus, and 2. virus entry in cell) to intracellular sub-nano disintegration (3. parasitic penetration in cellular biosynthesis, mediated by small molecular metabolites) to replicate viral macromolecules, which again nano-reintegrate toward new viral posterity (4. through self-assembly, maturation and delivery out cell in form of multiple reproduced new virions).

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So, the most preferable for nano-therapeutic intervention targets are:

1,4 – extracellular viral particles (virions), smallest, nano-scale organisms (20–300 nm);
2 – viral transformations during entry into cell, mediated by virus external sensors of 5–20 nm (earlier steps of viral life cycle), and
3 – self-assembling intermediates for new viral posterity maturation (late steps).

Free from any active metabolism serviced by small molecular species the molecular fundament of these targets consists of inter-bio-polymeric nano-complexes predominantly of protein (glycoprotein) nature, as well as of nucleic acids. The latter, covered in virions (1, 4) by the proteins, become accessible for direct molecular intervention within the targets of 2 or 3 type. Therefore, in view of the macromolecular level of

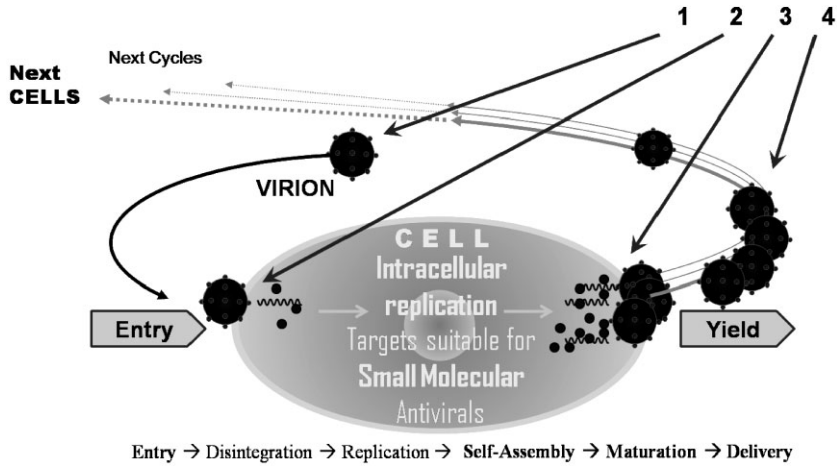


Figure 1.
A viral life cycle and targets for nano-therapy.

basic viral components, no traditional small molecular drugs, but just new polymeric forms of chemical compounds are prospective priority to development of the adequate nano-therapy against viruses.

Prediction of Fundamental Principles and Strategy for the Nano-Responsible Antiviral Agents Design

As a logic conclusion from the nature of viral life cycle, the first postulated basic principle for the purposed antiviral compounds design is **the nano-responsible macromolecular (polymeric) level**[1] in search for maximum of Index of Selectivity (IS):

$$IS = \frac{C_{50\%\ \text{CELL INHIBITION}\rightarrow\max}}{C_{50\%\ \text{VIRUS INHIBITION}\rightarrow\min}} >> 1 \rightarrow \max$$

$C_{50\%\ \text{CELL INHIBITION}}$, and $C_{50\%\ \text{VIRUS INHIBITION}}$ are the compound concentrations of the indicated effects.

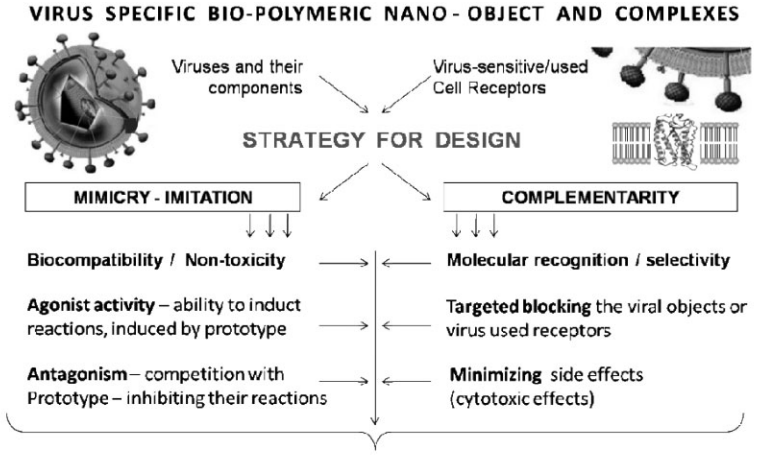
Where the IS is a measure of capacity to selective inhibition of the viruses (nano-objects) without detriment to cells (micro-objects).

This general principle in consolidation with other key principles has been formulated as fundament for the design strategy: "***From natural virus-specific bio-polymeric complexes toward artificial macromolecular antivirals***", represented on Fig. 2.

Previously Studied Basis, Immune Stimulating Polyelectrolytes

Early as a potential antiviral compound generation the synthetic polymeric mimickers of linear-alternating structure of nucleic acids (NA) backbone (the furan-derived and negative charged acidic species alteration) have been synthesized and studied from among the synthetic alternating copolymers of furan- and anion- genic monomers of carboxy-acidic type.[2,3] And really in accordance with the expected potency to agonistic activity these compounds (similarly to viral genome NA) possessed ability to induce strong antiviral immune response, at least through the induction of antiviral forms of interferon. This led to broad antiviral activity of these compounds due to immune adjuvant effects *in vivo*, against various viruses, including *eastern equine encephalomyelitis-, tick-born encephalitis-, rabies-, Crimean hemorrhagic fever-, meningoencephalomyelitis* viruses (Fig. 3) and other.[3,4]

However this antiviral activity was an action indirectly targeted to viruses,



for example the particular algorithms:

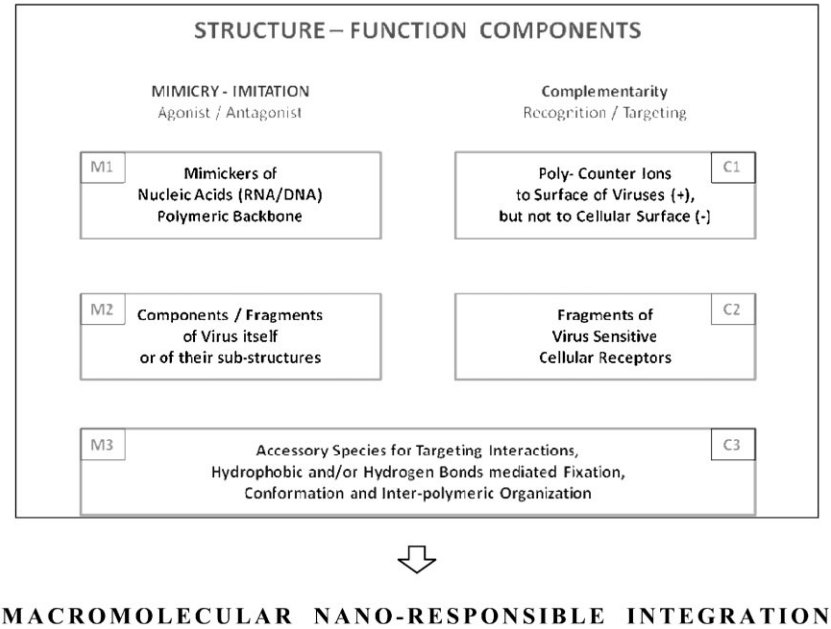


Figure 2.
The purposed design strategy: "From natural viral bio-polymeric complexes toward artificial macromolecular antivirals".

mediated through immune reactivity effects most evidently observed *in vivo* without any significant manifestation *in vitro*.

In frame of the explored strategy (Fig. 2) we predicted possibility to transform this polymeric platform toward bi-level active derivates, acting not only as antiviral immune stimulators (*in vivo*) but as directly targeted to viral nano-objects inhibitors (*in vitro*) too.[5,6] The required targeting has been assumed can to be achieved by complex and rational macromolecular integration of the structure-functional blocks, shown on Fig. 2. And these novel antiviral generations of the hybrid polymeric

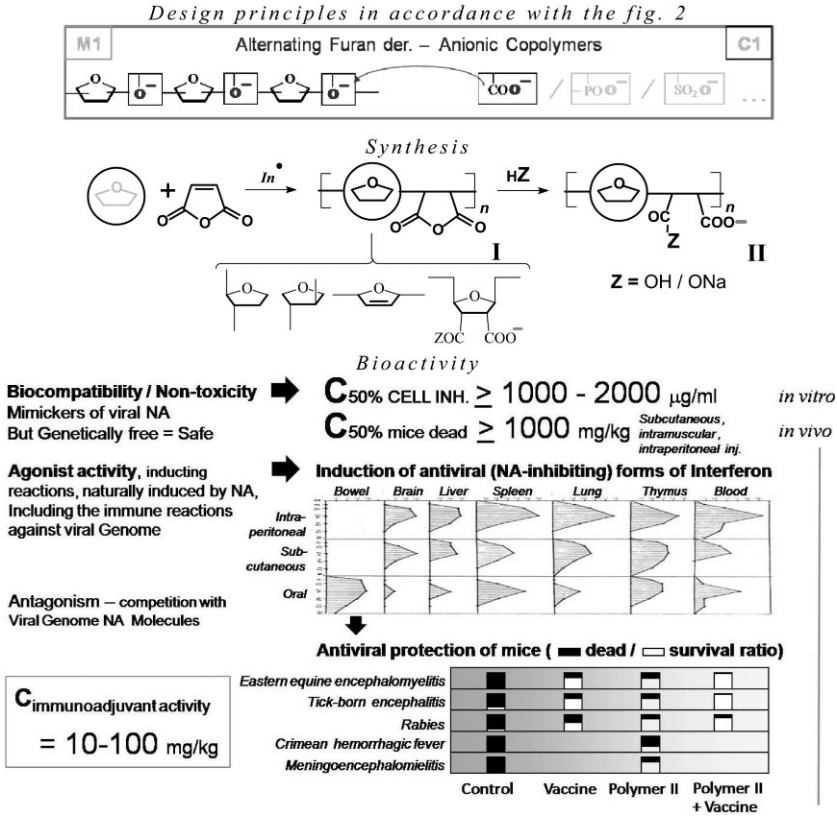


Figure 3.
An example of synthetic polycarboxyacid-based mimickers of nucleic acids backbone development for virus inhibition purposes.

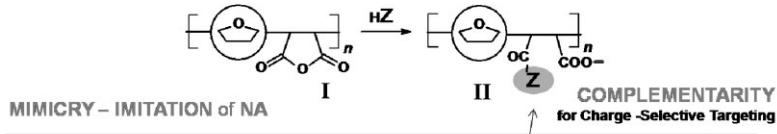
compounds was constructed first of all by graft-modulation of the early developed linear-alternating synthetic polyanions **II.** The high reactive poly-anhydride precursors **I** were used for purposed graft-modulation of side groups Z (Fig. 3), where Z = regulated combinations of species predesigned and synthesized, as M2/C1/C2/M3-C3 (Fig. 2) pharmacophores.

Electrostatic Factor for Activation of the Antiviral Potency

Enhancement of Charge-Selective Targeting by Graft-Regulated Side Groups (Z → C1 max)

The main planed task of this route of conversion was enhancement of electrostatic-selective complementarity to just virions, as counter (positively) charged nano-objects, without detriments for normal cells, as negatively charged (on external surface of plasma membrane) micro-objects. Maximum negatively charged macromolecules were assumed should to be most effective for electrostatic switching the viral attack from cells to the artificial macromolecules. As a relevant result, the neutralization of the viral particles by the polymeric compound, and preventing the virions adsorption/attachment to cell surface, was predicted.

For this purpose the synthetic polyelectrolyte **II** (Fig. 4) with slight anionogenic carboxyacid groups (-Z = -COOH, 100%) has been step-by-step converted toward strong anionic - sulfated derivatives **II** (-Z = -COOH → -CONH-X-SO_3^-). The enhanced interest to sulfates followed



Side group Z modulation mol. % ratio			$C_{50\%\ CELL\ INH.}$ µg/ml		IS = $C_{50\%\ CELL\ INH.}$ / $C_{50\%\ VIRUS\ INH.}$				
					HIV-1	Hyman Cytomegalovirus			
Z_2	OH / O⁻	SO_3^-	Lympho-blast	Fibro-blast	Prevent-Ther.ap.	Virucid	Microbicid	Prevent	Therap.
0	**100**	**0**	> 1 000	3 500	> 37	< 10	350	< 10	< 10
16	**84**	**0**	> 1 000	1 700	> 125	< 10	17	< 10	< 10
16	**72**	**12**	> 1 000	1 440	> 139	66	**1 400**	22	< 10
16	**69**	**25**	> 800	1 420	> 242	**355**	**1 400**	189	< 10
16	**34**	**50**	> 800	> 500	> 258	**5 000**	**500**	91	< 10
0	**20**	**80**	> 2 000	3 000	**> 680**	**7 500**	**5 500**	**250**	**4 286**

Anti-AIDS

Figure 4.
Enhancement of anionogenic potency of the polyanions II results in a strong amplification of antiviral efficiency (experimental models in vitro of the human immunodeficiency virus type 1 (HIV-1) and the human cytomegalovirus infections[7–11]).

from analysis of literature: 1) human cells heparan sulfate receptors are widely sensitive to viruses, including the human cytomegalovirus (HCMV), and 2) the sulfated tyrosine residues also play an essential role as active centers of chemokine receptors used by the human immunodeficiency virus type 1 (HIV-1).

As it shown on the Fig. 4 the electrostatic modulation of the macromolecules toward more and more negatively charged (sulfated) polyanions really leads to potent amplification of the antiviral-selective efficiency. As a result the new generation of antiviral macromolecular compounds **II** (-Z → -X-SO_3-) was developed.[7–9]

The recent evaluations under variable experimental models of viral infections of HIV-1 and HCMV evidently shown the enhanced antiviral effect exactly of the most sulfated derivate against both the HIV-1 and HCMV.[10] At the both viral infections the powerful antiviral inhibition was observed within earliest steps of virus entry into cells. Detailed study of the anti-HCMV effects at various experimental conditions confirmed a combination of several antiviral-protecting mechanisms, involving probably: 1) direct microbicidal neutralization of extracellular virus, 2) prevention of the virus adsorption/attachment to and entry into cells, and 3) post-entry effects of agonist/antagonist activity during intracellular steps of viral replication.[11] Thereby the electrostatic-selective modulation of the developed macromolecules leads to activation of a complex of diversified antiviral mechanisms. It should be postulated that just the combination of plural antiviral effects is most promising precondition to prevent a virus drug-resistance, the acute but naturally predetermined problem of the modern pharmacology based upon traditional drugs of small molecular (nano-inadequate) level.

In view of the fact that HIV-1 is direct infective factor, and HCMV is one of most dangerous co-factor of AIDS, the current experimental results provided a new macromolecular basis for creation of effective drugs/microbicides against HIV/AIDS + HCMV (sexually transmitted infections).[7–11]

Development of Other Side-Group Modulating Factors for Activation of the Antiviral Potency

The complex macromolecular design, as strategy for "***Poly-cooperation of ionic and non-ionic antiviral vectors***", has been formulated and discussed in our report on the 20th International Conference on Antiviral Research.[12] And for the present day this strategy was advanced in at least five generations, Fig. 5, of experimentally graft-modulated macromolecular compounds:

1) $-Z_1$ = charge-targeted anionic pharmacophores (for the M1/C1 blocks, Fig. 2),
2) $-Z_2$ = membrane-tropic alicyclic pharmacophores (for the M3/C3 blocks, Fig. 2),
3) $-Z_3$ = cholesten vectors to membrane "raft-"-domains (for the M3/C3 blocks, Fig. 2),
4) $-Z_4$ = HIV-1 gp120-sensitive fragments of cell receptors (for the M2 block, Fig. 2),
5) $-Z_5$ = HIV-1 capsid MA protein derived sub-peptides (for the C2 block, Fig. 2).

The preliminary study of the graft-modulating routes $1)+2)$,[13–21] $1)+3)$,[22] and $1)+2)+4)$[23–26] has been initiated by our research group early, and the route 5) is an exploration initiated recently.[27] Now these investigations are developed for nano-medicine purposes. For example present day summarized data of the side groups "Structure – Activity Relationship (SAR)" in respect with experimentally observed ranges of the IS for anti-HIV-1 activity is represented on Fig. 5. The essential potentiation of antiviral activity *in vitro* within the $Z_1 + Z_2$ –modulated generations against other viruses of influenza (A and B types), parainfluenza, respiratory, *herpes viridae* family has been achieved as well.[28,29]

Computation-Based Modeling and Analysis of the Present Obtained Database of the SAR within the Side-Group Modulating Factors

In relation with the discussed tasks the computer-based modeling and analysis have been initiated and carried out by our research group (in cooperation with Alexander Veselovsky laboratory from

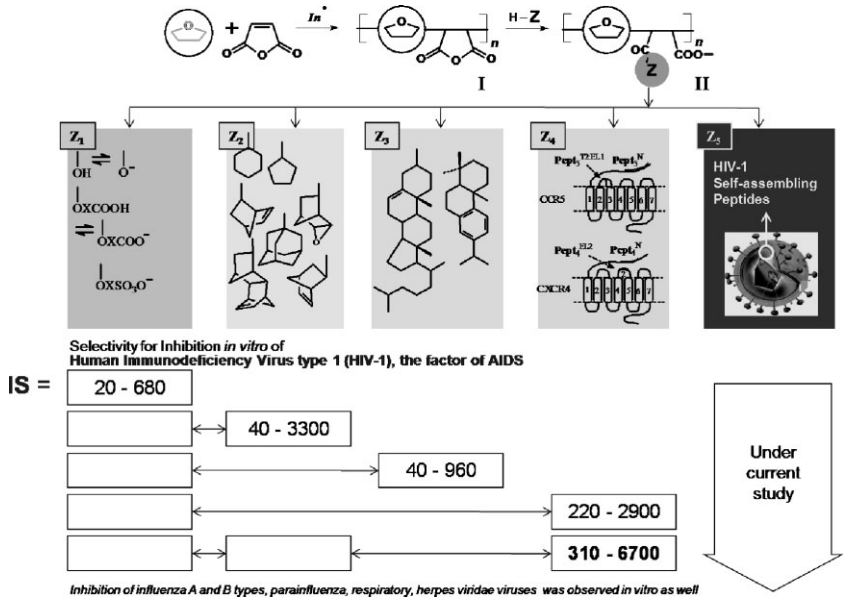


Figure 5.
Complex side groups modulation of the macromolecules **II** for amplification of antiviral efficiency. And current data of the IS evaluations of the corresponding macromolecular compound generations on experimental models of HIV-1 in vitro.

Institute of Biomedical Chemistry, Russian Academy of Medical Sciences). The experimentally accumulated in our group SAR database of "$Z_1/Z_2/Z_3$-modulated polyanions **II** structure – anti-HIV-1 activity *in vitro* at the entry steps" (included hundreds experimental courses) was pre-explored by variable docking and molecular dynamic techniques in search for:

a) the virus entry-key biopolymeric complexes as targets for the polymers **II** blockage,
b) the polymers **II** dominant fragments (factors), responsible for the required targeting,
c) the epicenters of the polymers **II** – viral biopolymeric nano-complexes interference,
d) the computation-based theoretical interpretation of the observed experimental data,
e) basic principles and methodology for the computation-predictive prognosis to design - synthesis of novel nano-selective virus-targeted macromolecular "robots".

First of all our efforts was focused on the starting events of HIV-1 infective life cycle, the entry in cells. Key bio-polymeric intermediates of this initial stage of virus intervention, as targets for therapeutic counter-intervention, have been observed and extracted from bibliography analysis of this problem, reviewed early.[5,30–32] The bio-polymeric factor, most crucial for the HIV-1 entry, is the HIV-1 envelope glycoprotein gp41 in activated state inducing the virus-cell fusion. A "bifurcation point" in the fusion initiation is the trimolecular $(gp43)_3$ self-aggregation, active epicenter of which is located within coiled-coil nano-complex [$(C\text{-domain})_3$-$(N\text{-domain})_3$ regions of the $(gp41)_3$], Fig. 6.

On base of known 3D structure of this complex we simulated series of computing-docking experiments to estimate partial targeting and free energy minimizing contributions of various chemical fragments, "structure factors", from the studied synthetic macromolecules **II.** As a preliminary step, the fragments from backbone and side-groups were pre-evaluated discretely and separately. On the next steps the computation-simulated derivates and combinations of these fragments step-by-step

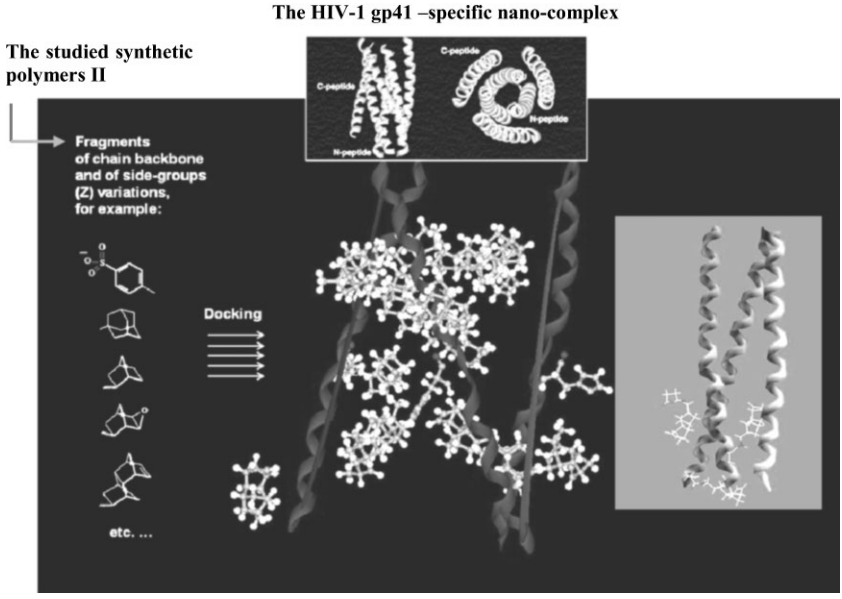


Figure 6.
The HIV-1 six-coiled $(N\text{-domain})_3$-$(C\text{-domain})_3$ nano-complex fragment of $(gp41)_3$, and examples of it docking evaluation as target for partial recognition-blocking by variable structure fragments from studying generations of synthetic polymers II(HMS).

approximated toward the macromolecular integrated structures were explored too.

The number of scoring functions (Chem-, D_-, G_-, F_-, PMF_- Score) and their consensus estimates, as well as more informative authoring criteria, has been applied for this aim. To the present day the following preliminary results were obtained: 1) the estimated ''structure factors'' (SF) were ranked in order of calculated [SF + viral target] complexes free energy minimizing, 2) location of the SF docking sites within the predominantly N-domain of the viral nano-complex was studied, and 3) correlations ''computed free energy – SF structure – anti-HIV activity *in vitro*'' was find too.

The noted correlation between the computation-based and *in vitro* experiments confirmed a high probability of the hypothesis that exactly HIV-1 envelope glycoprotein gp41 nano-complexes can be involved as targets to direct therapeutic interventions of the anti-HIV active HMS.

Among the computation-simulated sites for the blockage, as energetically most preferable, tree hydrophobic cavities, symmetrically located on the surface of the $(N\text{-domain})_3$ pre-complex were defined. As has been calculated these cavities to be more targetable for the cage hydrocarbons (norbornane-, adamantine-, and dinorbornane-kind) from the Z_2 variations (Fig. 5). Alternatively these cavities responded also to Z_1 interfere, if the spacer bridge (Fig. 5) –X–= –NH–(*para*-C_6H_4)–. Nearly the hydrophobic cavities the positive ionized residues of Lys were detected. This provided an additional stabilization of ion-counter ion interactions between the $\text{-}NH_3^+$ group of Lys and sulfoxyanions of sulfated Z_1 groups. H-bonds formation and specificities of the viral proteins conformation and topology have been also taken into consideration.

The computational simulation of these multifactor and multipoint interactions allowed us modeled the effects of the biofunctional synergism or antagonism between the heterogeneous Z_1 and Z_2 ''antiviral vectors'' in relation with them chemical configuration and macromolecular integration in HMS for maximum of the purposed antiviral selectivity. Totally the present docking results allow interpret the experimental data observed *in vitro*. And computation-modeling methodology will be used to predict most promising SAR for potent antiviral inhibitors of the HMS type.

Design and Synthesis of Novel Hybrid Macromolecules by Virus-Derived Fragments Co-Integration

The design of novel HMS with the side groups modulation by viral components ($-Z_5$, Fig. 5) has been tested recently in focus of the HIV-1 gag matrix protein p17 (MA). The MA plays an essential role in the HIV life cycle at earliest (viral uncoating, RNA delivery to nuclei) and latest (RNA re-transporting toward plasma membrane, virions nano-assembly-maturation) steps.[33] So, the MA, as promising anti-HIV therapeutic target, was included in priority of our anti-HIV inhibitors design strategy.[34,35] Experimentally we developed this idea (Fig. 7) in frame of: 1) MA-derived peptides (MAP) design and synthesis; 2) a cooperation of the MAP – MA interfere with an anti-RNA potency of the polymers II, expected from MAP grafted to the polymers II.

A number of MAP-imitators of MA helix 2–4 region fragments (responsible for MA-MA inter-self recognition-aggregation) were synthesized and modified to mono-amino group active reagents, suitable for single-linked grafting to the poly-anhydride precursor I. The corresponding conjugates of MAP with polymers I were synthesized and converted to the purposed MAP-polymers II. Finally, the obtained products were purified and prepared in lyophilized forms soluble in aqua (bio-)media. The grafting link location within amino acid chain or N-terminus of MAP was regulated by regioselective variation of the active and protected $-NH_2$ groups positions along the polypeptide chain. In parallel the fluorescent derivates of MAP and MAP-polymer I/II conjugates were prepared too.

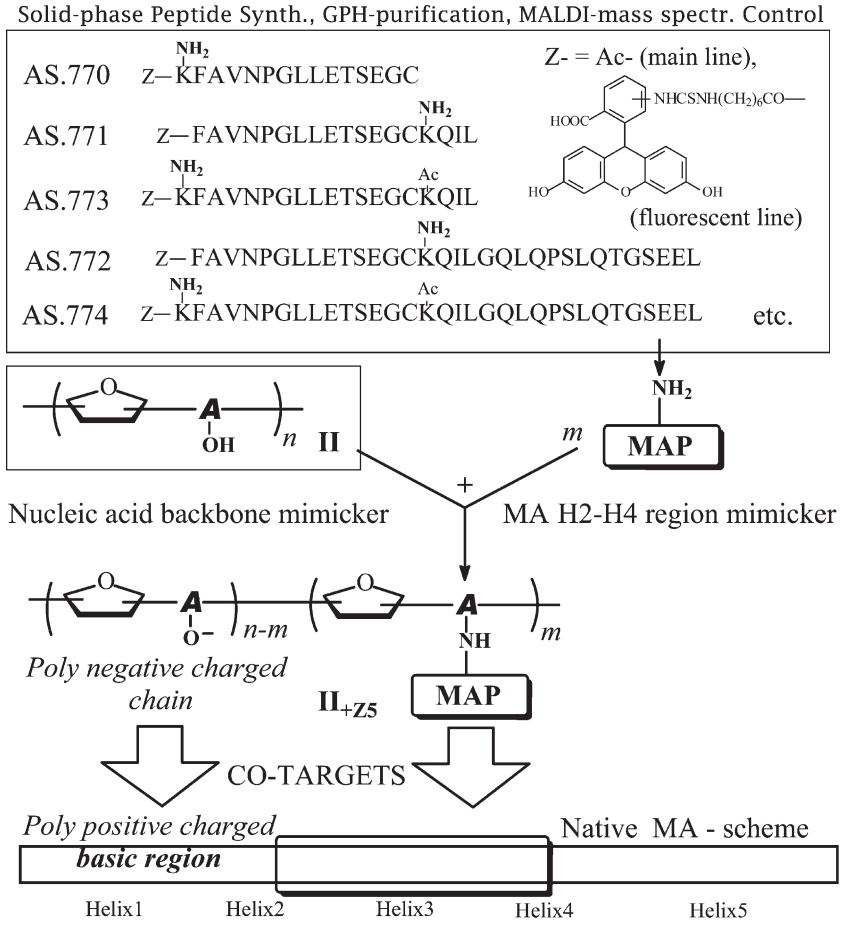


Figure 7.
The HMS constructed as polymers II derivatives modified by the polypeptide fragments of nano-responsible gag MA protein of HIV-1 capside.

The newly synthesized candidates (Fig. 7) to therapeutic counter-intervention in HIV life cycle by expected MA-interfering + RNA-antagonistic mechanisms are prepared for experimental evaluations *in vitro*. At the present time among the synthesized MAP the high active anti-HIV-1 inhibitor was really find already.[36]

New Graft-co-RAFT Macro Reagents for the Purposed Hybrid-Macromolecular Synthesis

To provide subsequent development of the above represented strategies for construction of the nano-selective macromolecular systems we advanced in creation of suitable macro-reagents, as precursors for the structure-functional components (blocks) required in accordance with Fig. 2. The experimental work was directed toward:

1. Multi-grafting polymeric templates I as high reactive (anhydride) precursors for the artificial NA mimetic agonist/antagonist II (Figs. 3–5, 7), counter-ion targeted to virions;
2. Mono-point graft-suitable reagents functionally designed as:

   2.1 Polypeptides from virus-sensitive cellular receptors[23–26] or viral proteins,[27]

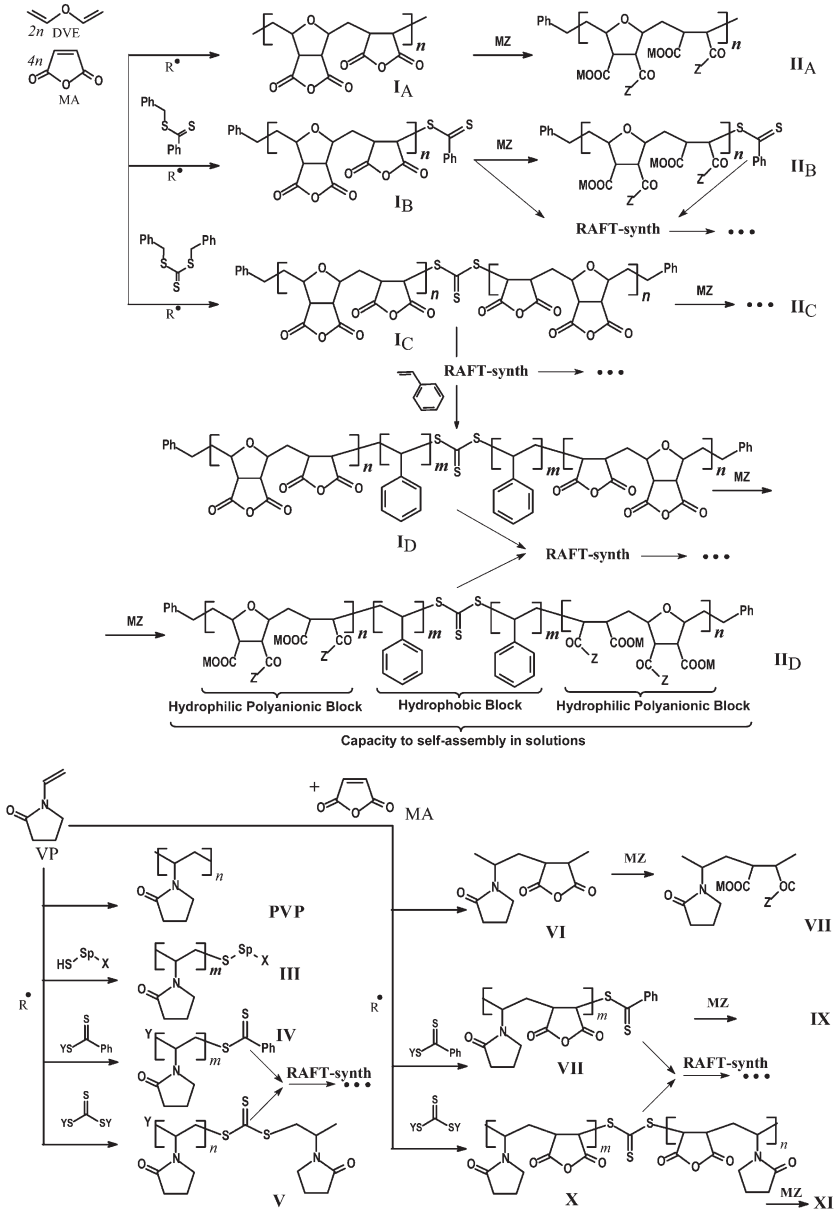


Figure 8.

The experimentally evaluated and developed synthetic routes toward mono-graft- (**III**), multi-graft ($\mathbf{I_A}$, **VI**), tail-RAFT-(**IV**), center-RAFT-(**V**), and novel graft-co-RAFT ($\mathbf{I_B}$, $\mathbf{I_C}$, $\mathbf{I_D}$, $\mathbf{II_B}$, $\mathbf{II_C}$, $\mathbf{II_D}$, **VII-XI**) reactive macro reagents for the purposed hybrid-macromolecular design and synthesis.

2.2 Synthetic polymeric agents for multiple H-bonds (protein-like) fixing,

2.3 Other functional species for the side groups/branches (Z) of II modulation;

3. Polymeric chain variable templates included reactive centers for pseudo-living radical (homo-/co-) polymerization by reversible addition-fragmentation chain transfer (RAFT) mechanism;

4. Combination of the 1 and 3 reactivity toward novel, graft-co-RAFT macro reagents.

The corresponding aspects of the RAFT-controlled and -reactive polymeric products synthesis were initiated by our research group and undertook in cooperation with colleagues from the Moscow State University (A. Zezin, E. Chernikova et al.). Some, most prospective routes for the synthesis and the yielded polymeric macro reagents are demonstrated on Fig. 8.

The novel ''Graft-co-RAFT Macro Reagents'' synthesis, as platform for homo- or co-polymeric products of random, alternating, block-, or gradient types with narrow MMD controlled parameters, was in part preliminarily reported on the last EPF'09 Congress.[37]

The developed methodology of combinatory macromolecular construction and obtained macro reagents could be used for advance toward biomedical (and more) applicable macromolecular products, including nano-responsible and/or self-assembling polymeric systems.

Conclusion

The represented investigations resulted in:

1) Development of new productive strategies for molecular design and purposed synthesis of bio-selective polymeric systems targeted to virus-specific nano-objects without toxic detriments for cells (micro-objects);
2) Creation of the novel generations of antiviral compounds for high effective binary inhibition of both human immunodeficiency virus and cytomegalovirus (basis for anti-HIV/AIDS and sexually transmitted co-infections prevention / therapy);
3) Design and synthesis for new hybrid polymeric compounds based on virus-targeted polyanions in combination with nano-structuring fragments of HIV-1 matrix gag protein;
4) Development of new macro reagents for combinatorial (Graft- + RAFT) hybrid polymeric synthesis, suitable for purposed construction of nano-responsible and bio-selective polymeric products.

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