University of Warsaw

Faculty of Chemistry

**Marzena Jankowska - Anyszka**

# Summary of scientific achievements in relation to habilitation procedure

Warsaw, September 2012

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## 1. FIRST NAME, LAST NAME

Marzena Jankowska-Anyszka

## 2. HELD CERTIFICATES, DEGREES – BY NAME, PLACE, AND THE YEAR OF RECEIVING, THE TITLE OF DOCTORAL THESIS

Doctor of chemical sciences, Warsaw, 04/17/2000

| | |
|---|---|
| Title: | "Synthesis, physicochemical and biological studies on analogues of the 5' end of mRNA and U snRNA" |
| Supervisor: | Prof. dr hab. Andrzej Temeriusz |

## 3. INFORMATION ON ACADEMIC EMPLOYMENT

| | |
|---|---|
| 1988 – 2000 | Warsaw University, Faculty of Chemistry, scientific technical worker |
| 2000 – | Warsaw University, Faculty of Chemistry, assistant professor (adjunct) |

## 4. BIBLIOGRAPHIC SUMMARY OF SCIENTIFIC ACHIEVEMENTS

| | |
|---|---|
| Total number of publications | 44 |
| Total number of publications after Ph.D. | 27 |
| Total Impact Factor of publications | 181 |
| Total Impact Factor of publications after Ph.D. | 102 |
| Total number of citations (without self-citations) | 951 |
| Hirsch Index | 16 |

Series of publication related to the main scientific achievements consists of 8 publications (total impact factor 33).

## 5. THE MAIN SCIENTIFIC ACHIEVEMENT RELATED TO THE ARTICLE 16 PARAGRAPH 2, FROM THE ACT OF 14 MARCH 2003 ON THE ACADEMIC DEGREES AND ACADEMIC TITLE, AS WELL AS THE ART DEGREES AND TITLE

### 5.1 Title of the achievement

Synthesis of 5' end mRNA analogues crucial for research on mechanisms of eukaryotic gene expression

### 5.2 List of selected publications related to the achievement

H1. Worch R, **Jankowska-Anyszka M**, Niedzwiecka A, Stepinski J, Mazza C, Darzynkiewicz E, Cusack S, Stolarski R. (2009): Diverse role of three tyrosines in binding of the RNA 5' cap to the human nuclear Cap Binding Complex, Journal of Molecular Biology 385, 618-627. IF 3.871

H2. Benarroch D, **Jankowska-Anyszka M**, Stepinski J, Darzynkiewicz E, Shuman S. (2010): Cap analog substrates reveal three clades of cap guanine-N2 methyltransferases with distinct methyl acceptor specificities, RNA 16 (1): 211-220. IF 6.051

H3. **Jankowska-Anyszka M.***, Piecyk K., Samonina-Kosicka J. (2011): Synthesis of a new class of ribose functionalized dinucleotide cap analogues for biophysical studies on interaction of cap-binding proteins with the 5 ' end of mRNA, *Organic and Biomolecular Chemistry* (15): 5564-5572. IF 3.696

H4. **Jankowska-Anyszka M.***, Piecyk K. (2011): Dinucleotide cap analogue affinity resins for purification of proteins that specifically recognize the 5 ' end of mRNA, *Bioorganic and Medicinal Chemistry Letters* 21 (20): 6131-6134. IF 2.554

H5. Liu W.Z., **Jankowska-Anyszka M.**, Piecyk K., Dickson L., Wallace A., Niedzwiecka A., Stepinski J., Stolarski R., Darzynkiewicz E., Kieft J., Zhao R., Jones D.N.M., Davis R.E. (2011): Structural basis for nematode eIF4E binding an m(2,2,7)G-Cap and its implications for translation initiation, *Nucleic Acids Research* 39 (20): 8820-8832. IF 8.026

H6. Piecyk K., Davis R.E., **Jankowska-Anyszka M.*** (2012): 5´-Terminal chemical capping of spliced leader RNAs, *Tetrahedron Letters* 53: 4843-4847. IF 2.683

H7. Piecyk K., Davis R.E., **Jankowska-Anyszka M.*** (2012): Synthesis of $^{13}C$- and $^{14}C$-labeled dinucleotide mRNA cap anlogues for structural and biochemical studies, *Bioorganic and Medicinal Chemistry Letters* 22: 4391-4395. IF 2.554

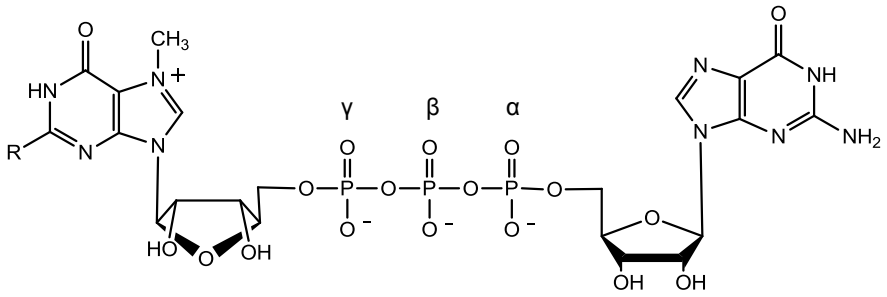
H8. Piecyk K., Davis R.E., **Jankowska-Anyszka M.*** (2012): Synthesis of $N^2$-modified 7-methylguanosine 5'-monophosphates as nematode translation inhibitors, *Bioorganic and Medicinal Chemistry* 20: 4781-4789. IF 2.921

## 5.3 Description of the achievement (aims, results and significance)

(*publications with the letter H are related to the list of publication on page 3, publication cited with the letter D are related to the list of publication after the Ph.D. degree on page 22)*

### An overview of the topic

The majority of eukaryotic RNA polymerase II transcripts, therefore mRNA (messenger RNA) and U snRNA (small nuclear RNA rich in uridine), possess at their 5' end a characteristic structure known as a “cap’. It consists of a 7-methylguanosine linked *via* an unusual for nucleic acid 5',5'-triphosphate bridge to the first transcribed nucleotide ($m^7$GpppN, where N is any nucleotide; monomethyloguanosine cap, MMG cap). In nematodes (e.g. *Caenorhabditis elegans*, *Ascaris suum*) however over 70% of mRNAs have a hypermethylated version of the cap with two additional methyl groups at the $N^2$ position of guanosine ($m_3^{2,2,7}$GpppN, where is N - any nucleotide; trimethyloguanosine cap, TMG cap).




R=$NH_2$, monomethylguanosine cap, MMG
R=$N(CH_3)_2$, trimethylguanosine cap, TMG

**Fig. 1** Structure of the 5’ end of mRNA (cap structure)

MMG cap is formed in the nucleus at the early stage of transcription by three successive enzymatic reactions: removal of a 5'-terminal γ-phosphate from oligonucleotide, transfer of GMP from GTP with formation of a 5’,5'-triphosphate bridge and a methyl group transfer catalyzed by (guanine-N7) methyltransferase. The resulting MMG cap plays important roles at many different stages of gene expression, which are performed through its interaction with various proteins. In the nucleus the cap is recognized by a cap-binding complex (CBC) and this interaction is involved in pre-RNA maturation by activating pre-mRNA splicing that helps to create the 3' end of mRNA. CBC complex is also involved in intracellular transport of small

nuclear snRNAs (uridine-rich) that are hypermethylated in the cytoplasm within the cap structure forming a trimethylated cap that serves as a signal for import of U snRNP ribonucleoprotein complexes (U snRNA + protein) back to the nucleus.
In the case of mRNA their MMG cap structure is recognized in the cytoplasm at an early stage of protein translation initiation by eIF4E (eukaryotic initiation factor 4E), and mRNA is recruited to the 43S initiation complex which is necessary for the polypeptide chain biosynthesis. Recognition of the mRNA cap by eIF4E is the critical, rate limiting step for efficient translation initiation, and it is a major target for translational control. Another important function of the cap is a protection of the mRNA against the 5'-exonucleolytic degradation.
As the cap is crucial in various cellular processes and it participates in the control of gene expression, fate of this structure must be strictly controlled. There are two main pathways of mRNA degradation ( 5'->3' and 3'->5' pathways) involving the cap breakdown by DcpS enzyme (degradation from the 3' to 5' direction, the enzyme hydrolyses the 5',5'-triphosphate bond between β and γ phosphate of the $m^7$GpppN), or by Dcp1/Dcp2 (degradation from the 5' to 3' direction, the Dcp2 enzyme cleaves the 5'-5' bridge between the α and β phosphate).
Ever since the discovery of the cap in the 70s, numerous studies have been performed to elucidate the molecular mechanism of various processes for which a cap structure is critical and to exploit the possibility of eIF4E as a target for anticancer therapy. It has been shown that eIF4E is a potent oncogene and its overexpression is associated with a variety of human cancers. Consequently, cap analogs as specific inhibitors to counteract elevated eIF4E level in tumor cells have been explored.

In most of that studies synthetic analogs of the 5' end of mRNA were intensively explored. Initially, the simplest mononucleotide analogues: 7-methylguanosine-5'-monophosphate ($m^7$GMP), 7-methylguanosine-5'-diphosphate ($m^7$GDP) and 7-methylguanosine-5'-triphosphate ($m^7$GTP) were used but with time and growing knowledge more complex analogues (e.g. dinucleotides such as $m^7$GpppG), modified within different parts of the cap structure were synthesized. Such compounds were used to characterize protein-cap interactions using structural (X-ray crystallography, nuclear magnetic resonance spectroscopy) and biophysical methods (fluorescence quenching, isothermal titration calorimetry) and to clarify the biological role of these interactions by molecular biology methods (*in vitro* and *in vivo*). Examples of different synthetic cap analogues designed and prepared by myself in terms of such studies and their application will be described later (section 6).

Based on interdisciplinary research involving chemists, molecular biologists and biophysicist, the knowledge about the processes of gene expression and mechanisms of their regulation significantly increased. At the same time many new cap-binding proteins were discovered. The most important were isoforms of eukaryotic translation initiation factor 4E from *C. elegans*, that exhibit diverse

specificity for both cap structures (MMG and TMG caps). To emphasize the fact that some eIF4E isoforms (eg. *Caenorhabditis elegans* protein IFE-3 and IFE-4) recognize only the MMG cap, and other (such as IFE-1, IFE-2, IFE-5 in *C. elegans* or *Ascaris suum* eIF4E-3) bind both MMG and TMG caps, they were named as dual specificity proteins. Considering that all mentioned isoforms show a high degree of homology with the human and mouse eIF4E protein and with each other, simultaneously having no systematic differences in amino acid sequences essential for the interaction with the cap, the varied isoform specificity still remains a puzzle. It is well known that all eIF4Es from higher eukaryotes strongly prefer MMG over TMG cap. Other newly discovered proteins are: (guanine-N2) methyltransferases isolated and preliminarily characterized in various organisms that are responsible for methylation of the exocyclic amino group during the synthesis of a TMG cap or nematode decapping enzymes, DcpS, which differ in substrate specificity from other organisms.

From the point of view of an organic chemist preparation of new synthetic cap analogues is highly connected with results of previous studies. New questions about the basis of dual specificity of nematode eIF4E isoforms, the biological role of this phenomenon and mechanism of methylation by (guanine-N2) methyltransferase have become for me an inspiration for designing and preparation of new chemical tools that allow to carry out specific studies with the participation of new proteins.

## Basic information about synthesis of cap analogues

Various synthetic analogs of the 5' end of mRNA have been extensively used in studies that are carried out for almost four decades. At the beginning the simplest mononucleotide analogues ($m^7$GMP, $m^7$GDP, $m^7$GTP) were used but with time and growing knowledge more complex analogues (eg. dinucleotides such as $m^7$GpppG), modified within different parts of the structure were synthesized. Although, many synthetic methods were developed, only some of them turned out to be universal and could be applied to a variety of nucleosides or nucleotides (e.g. phosphorylation at position 5' nucleoside using Yoshikawa method). Due to this, there is a large group of techniques that work better, or worse in specific cases (e.g. there are many methods of preparation of nucleoside 5'-triphosphates, but none of them is universal). Usually the synthesis of a particular compound requires therefore trying out various methods and sometimes their significant modification and/or optimization.

Another difficulty to overcome is the solubility of nucleotides. As it is generally good in water, but poor in organic solvents, it requires time-consuming procedures to transfer nucleotide into organic salts. Ammonium salts are often employed and they are formed in the reaction with the tertiary amines such as tri-n-octylamine, tri-n-butylamine or triethylamine. Another factor that also reduces the solubility of cap analogues in organic solvents is a positive charge on the imidazole ring of the guanine that is generated by a methyl group at the N7 position. A further difficulty to

overcome is the reduced stability of compounds containing 7-methylguanosine. It is known that the imidazole ring in an alkaline solution is degraded, whereas an acidic conditions decompose N-glycosidic bond connecting the nitrogen base with the sugar. Isolation and purification of intermediates and final products, during the cap analogue synthesis is usually time-consuming and troublesome. The obtained reaction mixtures are complex and contain compounds that usually possess similar physico-chemical properties, which not allow to use simple methods to isolate the product for example precipitation, crystallization or extraction.

Separation of the desired compound almost always requires the use of chromatographic methods such as: adsorption chromatography on silica gel (in the case of nucleoside derivatives) or ion-exchange chromatography on DEAE-Sephadex (in the case of nucleotide). The resulting products often do not exhibit the desired purity and are subjected to additional purification. Nowadays high performance liquid chromatography (HPLC), on reversed phase or ion exchange HPLC is the method of choice. An important factor in chromatographic methods is the eluent selection. It is crucial to obtain the final compounds in the form of organic salts, to be able to remove salt excess from the solution after separation. For this reason, we use triethylammonium bicarbonate, ammonium bicarbonate and ammonium acetate based buffers, which can be easily removed by distillation under reduced pressure or by repetitive lyophilization. Prepared in this manner cap analogues before they are used in various biophysical and biological studies are transferred into sodium salts and identified by high resolution mass spectrometry and $^{1}H$ and $^{31}P$ nuclear magnetic resonance.

**Overview of specific aims and results**

Recently the growing importance of biophysical techniques in variety of studies on the nature and thermodynamic or kinetic characterization of protein-ligand interactions in solution is observed. Some of these methods identify interaction of target molecules with specially prepared ligands bound to the matrix (e.g. surface plasmon resonance SPR). Others use neutral markers - for example in electron spin resonance (ESR), nonreactive radicals - attached to specific sites of the biological molecules thus providing information of probes environment. All such methods are suitable to gain a comprehensive insight into the dynamic nature of the interaction of the RNA 5’ terminus with proteins. To meet these needs, I decided to design and develop a method to synthesize a new class of dinucleotide cap analogues bearing various molecules connected within the cap structure which will be an excellent tool for cap–protein interaction studies. Such modification cannot interfere the basic structural elements of the cap that are responsible for protein binding.

Based on previous results, for functionalization of the dinucleotide cap analogue, I chose, the least involved in the interaction with protein, sugar moiety of the second base. As the 2',3'-*cis* diol group present in sugars e.g. in ribose moiety

forms acetal easily, I decided to use for a cyclization step levulinic acid having apart from the carbonyl group (necessary to form the acetal) also the carboxyl group, which would allow to incorporate another molecules e.g. molecular probes.

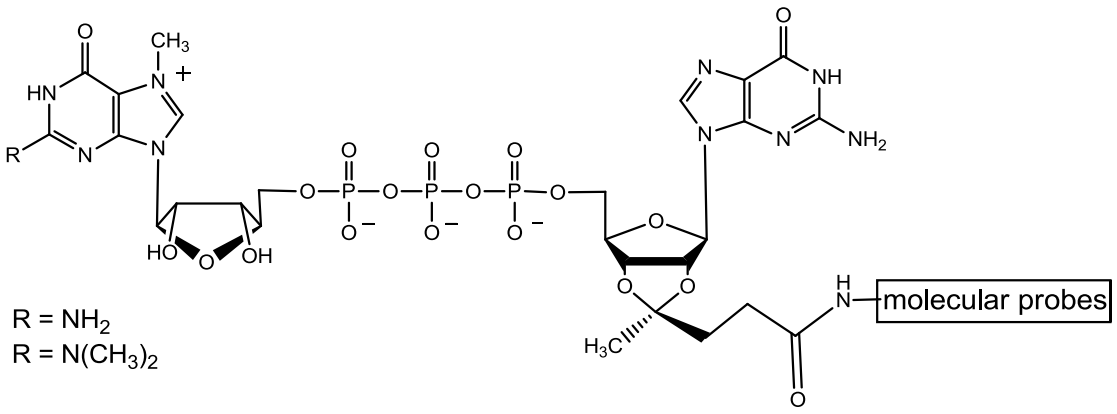



**Fig. 2** Ribose functionalized dinucleotide cap analogues

Publication [**H3**] presents a synthesis of the first dinucleotide cap analogues with attached various ligands and markers (Fig. 2). The paper describes methods such as: guanosine functionalization with ethyl levulinate, 5'-phosphorylation and coupling reaction with a second nucleotide ($m^7$GDP or $m_3^{2,2,7}$GDP) leading to levulinate modified dinucleotide cap analogues ($m^7GpppG_{Lew}$, $m_3^{2,2,7}GpppG_{Lew}$). In the case of MMG cap analogue ($m^7GpppG_{Lew}$) four synthetic routes using different strategies (activation of $m^7$GDP and coupling with LewGMP or vice versa) and coupling conditions (aqueous or anhydrous) were described. It was also shown that only one route leads to an analogue of the TMG cap ($m_3^{2,2,7}GpppG_{Lew}$) (LewGMP activation and coupling with a $m_3^{2,2,7}$GDP in anhydrous conditions) with a satisfactory yield. At the same time, presented method seems to be the most universal, as it requires development of activation conditions only once, for LewGMP (the compound can be potentially activated not only within the phosphate but also the carboxyl group). The second part of the paper describes coupling reactions of the carboxyl group present in the MMG and TMG cap analogues ($m^7GpppG_{Lew}$, $m_3^{2,2,7}GpppG_{Lew}$) with an amino group of variety molecules, e.g.: ethylenediamine (can be used either for further chemical reactions or directly for the immobilization of the cap analogue to the surface, chip, etc.), biotin (which forms a complex with avidin and streptavidin, which is used widely in many techniques) and 4-aminoTEMPO (a stable radical that can be used in electron spin resonance spectroscopy). It should be also noted that proposed method for functionalizing dinucleotide cap analogues is universal and allows to introduce within the cap structure variety of other compounds (e.g. fluorescent probes).

One of the analogue, the MMG cap functionalized with levulinic acid with attached ethylenediamine ($m^7GpppG_{Lew\text{-}EDA}$) was used to study its interaction with the

nuclear cap-binding complex CBC using a surface plasmon resonance (SPR) [**H1**]. This heterodimer protein recognizes, as its cytoplasmic counterpart, factor eIF4E, MMG cap and through this interaction it plays a key role in many processes of gene expression (including pre-mRNA splicing, polyadenylation of the 3' end of mRNA, transport of mRNA and U snRNA to the cytoplasm). The biophysical basis of the molecular mechanism of recognition of the cap by CBC were the main purpose of the research conducted in Prof R. Stolarski (Department of Biophysics, University of Warsaw) group, which I collaborate with for years. International Institute of Molecular and Cell Biology in Warsaw bought at that time a Biacore apparatus which opened the possibility of using SPR technology and undertake joint research, during which I had the opportunity to use my experience acquired during one of visits in the Prof. Robert Rhoads laboratory (LSUHSC, Shreveport, USA). As the CBC protein requires for efficient binding the presence of the second base in the cap srtucture, it was necessary to prepare a dinucleotide cap analogue for the immobilization on the chip sensor [D10]. This was achieved by functionalization of a dinucleotide cap with levulinic acid which was further coupled with ethylenediamine. The free amino group was used for immobilization on the sensor surface in the form of carboxymethylated dextran. Kinetic measurements of binding of CBC and its mutants to surface immobilized $m^7$GpppG derivative ($m^7GpppG_{Lew\text{-}EDA}$) by means of SPR were performed. Based on these studies association/dissociation constants of CBC binding to the cap were determined and provided important clues for a kinetic model of the CBC–cap association.

The idea of levulinate functionalized cap analogues was also used to prepare a new class of affinity resins based on dinucleotide cap analogues. Affinity chromatography has been used for years for the isolation and purification of cap-binding proteins. Mononucleotide based resins, $m^7$GTP and $m_3^{2,2,7}$GTP-Sepharose, as it will be shown in section 6, have been crucial to characterize newly discovered proteins. Unfortunately, these affinity resins may be insufficient in the case of proteins that strongly interact with a second base of the cap. An example of such protein is the nuclear cap binding complex (CBC). For isolation and purification this kind of proteins I developed a strategy for immobilization of dinucleotide cap analogues on the Sepharose bead [**H4**]. The idea was based on functionalization of the cap within the sugar moiety with levulinic acid and usage of the remaining carboxyl group to join it with a hexamethylenediamine linker from the resin surface (Fig. 3). Linker was used in order to increase the distance of the ligand from the matrix and to facilitate access of potential proteins. In this way new affinity resins with MMG and TMG caps ($m^7$GpppG- and $m_3^{2,2,7}$GpppG-Sepharose) and their equivalents having a bridging methylene group in the 5',5'-triphosphate chain between β and γ phosphate were prepared. The purpose of this last modification was to increase the resistance of the ligand to hydrolysis agaist DcpS enzymes. This modification seems to be very useful in the case of isolation of proteins from cell extracts. It is worth to mention that prepared affinity matrices are the first dinucleotide based resins, with MMG and TMG cap analogues, additionally strengthen by bridging

modification that prevents their degradation. In the paper the use of obtained affinity resins to purify *C. elegans* IFE-5 isoform was also described.

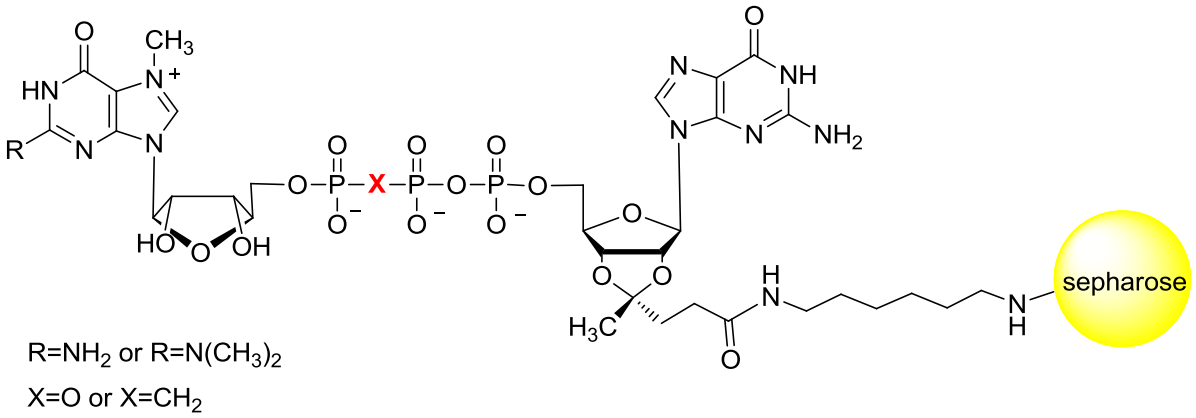



**Fig. 3** Dinucleotide cap analogue affinity resins

Trimethylated cap (TMG cap) is present at the 5' end of the majority of mRNA population in nematodes such as *Caenorhabditis elegans* and *Ascaris suum* and is added with a conseved 22 nt spliced leader sequence (SL) during *trans*-splicing. As it was indicated in [D6], [D13], [D17] papers the spliced leader sequence decisively enhances translation of TMG-capped RNAs, having little effect on translation of MMG-capped messages *in vitro* and *in vivo*. This synergistic interaction of the TMG cap and the SL sequence was called the "SL effect". It is highly likely that in nematodes, except proteins that interact with a TMG cap of mRNA (eIF4E isoforms of *C. elegans* described in [D1], [D2], *A. suum* eIF4E-3 [D6], *C. elegans* and *A. suum* DcpS [D5]) other unknown proteins that are responsible for the synergistic effect of the SL and the TMG cap structure are also present. In order to carry out these binding studies, an affinity resins with the ligand consisting of 22-nucleotide sequence with a TMG cap at the 5' end, would be an invaluable tool. Due to synthetic reasons and universality (possibility to use this compound in other studies, such as the interaction of eIF4E protein with eIF4E isoforms by SPR) I decided to use a commercial oligonucleotide with a biotin at the 3' end. This modification allows the immobilization of the ligand on the surface functionalized with streptavidin.

Synthesis of capped-oligonucleotides is troublesome for the organic chemists. Although, numerous studies have been carried out to identify an efficient method to synthesize capped oligonucleotides any of them is universal. Some are based on solid supported methods, some are 'in solution" but they are mainly applied to MMG cap or even to its simplified structure (5' terminal capping via more stable 5',5'-diphosphate bridge). Usually short oligonucleotides were used and in some cases the final product purification was not presented. This issue is very important as pure compounds are essential for the use in biophysical or biochemical studies. Because of only slight differences in the physicochemical properties of capped and uncapped oligonucleotides, their separation and purification is a serious problem. That is why my goal to obtain a set of 22 nt oligomers capped at the 5' end with either MMG or

TMG cap was extremely challenging. In [**H6**], studies on determining a suitable method of coupling in solution and optimization of reaction conditions which resulted in a significant simplification of the procedure of separating the products from the reaction mixtures, are described. Conditions of final products purification using ion-pair reversed-phase high performance chromatography (IP RP HPLC) are presented as well. In addition to oligonucleotides biotinylated at the 3' end and capped with $m^7$GpppG, $m_3^{2,2,7}$GpppG and GpppG at the 5' termini, compounds based on a 22-nucleotide chain without biotin for structural studies were also prepared. Such studies require milligram amounts of capped-oligonucleotides that cannot be obtained by *in vitro* transcription.

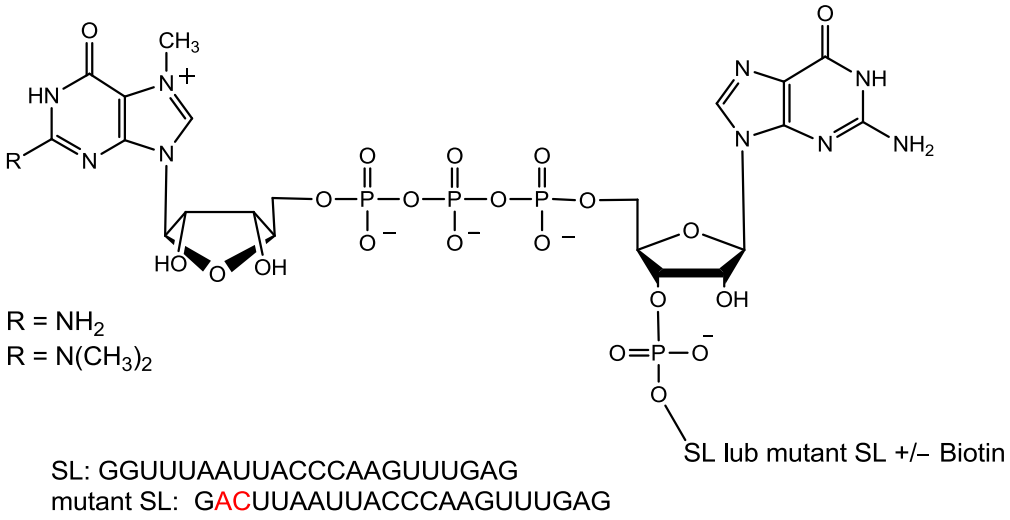



**Fig. 4** 5'-capped spliced leader RNAs

The most recent findings indicated that the conserved 22 nt SL sequence and structural elements within a spliced leader including a small stem-loop are required for efficient translation of TMG-capped RNAs. In order to verify this hypothesis and to confirm the creation and the role of the loop in the SL it become crucial to prepare oligonucleotides with introduced mutations that will disrub the potential base pairing within the SL stem-loop. Synthesis of a set of six compounds containing the mutation within the SL sequence capped with MMG or TMG cap at the 5' end and 3' biotinylated (Fig. 4), surprisingly turned out to be much more difficult. Changing only two nucleotides in the 22-nucleotide sequence resulted in a significant lower coupling efficiency together with problems with oligonucleotide purification. The paper discuss possible reasons for such situation.

In conclusion, I prepared twelve new compounds, MMG and TMG capped oligonucleotides with the longest sequence published so far. They are key tools for the reaserch that is aimed at elucidation of the mechanism of translation of the TMG capped mRNA in nematodes using different biophysical and biochemical methods (SPR, fluorescence quenching, ITC, NMR, crystallography, "pull down" assay).

These experiments are currently being conducted in the Dr Richard Davis laboratory (University of Colorado School of Medicine, USA).

One of the compounds (TMG-SL) was already used together with other analogs that I synthesized ($m_3^{2,2,7}$GTP, $m_3^{2,2,7}$GpppG) to compare the binding of these three ligands with *A. suum* eIF4E-3 by NMR chemical shift perturbation analyses [**H5**]. The binding of $m_3^{2,2,7}$GpppG-SL may contribute to the structure or interactions within the cap-binding pocket and may influence *Ascaris suum* eIF4E-3 interactions with other translation factors through protein conformational changes In this paper dealing with interaction of eIF4E-3 protein with a TMG cap (the long-standing collaboration with Dr Richard Davis), it was also disccused: 1) quantitative results on protein interaction with various cap analogues by protein fluorescence quenching titration by cap analogues (performed by myself in collaboration with Dr Anna Niedźwiecka, the Environmental Laboratory of Biological Physics, Institute of Physics, Polish Academy of Sciences, who developed this method) and isothermal titration calorimetry (e.g. $m_3^{2,2,7}$GTP, $m_3^{2,2,7}$GpppG), 2) comparison of binding affinity of *A. suum* eIF4E-3 protein with human eIF4E using affinity chromatography, mainly $m^7$GTP- and $m_3^{2,2,7}$GTP-Sepharose and 3) the crystallographic structure of eIF4E-3 in complex with a mononucleotide cap analogue ($m_3^{2,2,7}$GTP).
In order to verify the earlier hypothesis [D1], that steric hindrance, associated with presence of two methyl groups within the guanosine exocyclic amino group of the TMG cap ($m_3^{2,2,7}$GpppG) is responsible for the reduced affinity of eIF4E from higher eukaryotes to a TMG cap, the analog having a large benzyl substituent at the $N^2$ position was synthesized ($bzl^2m^7$GMP) as described in [**H8**]. This compound was used to determine the binding affinity of two proteins (*A. suum* eIF4E-3 and human eIF4E) by ITC and by *in vitro* competitive translation inhibition assay in *A. suum* extract and rabbit reticulocyte lysate. These studies revealed that both proteins exhibit a 10-fold increased affinity to $bzl^2m^7$GMP analog compared to $m^7$GMP, suggesting that steric hindrance around the exocyclic amino group does not prevent protein binding. More interestingly it turned out that $bzl^2m^7$GMP is a more effective inhibitor of translation in the *A. suum* extract than $m^7$GTP that inhibits strongly translation in other translational systems.

Another tools that were prepared to study cap-protein interactions were $^{13}$C- and $^{14}$C-labeled dinucleotide mRNA cap analogues. $^{13}$C and $^{14}$C labeled mono and trimethylated cap analogues will facilitate analysis of the specificity of nematode eIF4E isoforms for the MMG and TMG caps and will be extremely useful as tools for NMR studies ($^{13}$C), for monitoring chemical and enzymatic reactions ($^{14}$C), or to synthesize RNA containing the $^{14}$C label by *in vitro* transcription. Paper [**H7**] describes a simple and short method for specific labeling of mono- and trimethylated caps with $^{13}$C and $^{14}$C isotopes. The labels were introduced within the cap structures either at the N7 for monomethylguanosine cap or N7 and $N^2$ position for trimethylguanosine cap. Nevertheless, for the synthesis of $^{13}$C /$^{14}$C MMG and TMG caps, the procedure was optimized in order to minimize the number of steps with the

radioactive materials and due to the cost of isotopically labeled substrates ($^{13}C$ or $^{14}C$ methyl iodide).

Results described in paper [**H5**] indicated that $bz^2m^7GMP$ binds *Ascaris suum* eIF4E protein with a 10-fold higher affinity compared to $m^7GMP$ and is a strong inhibitor of either a $m^7G$- or $m_3^{2,2,7}G$-capped mRNA translation in a cell-free *Ascaris suum* translation system. Many nematodes, including *Ascaris suum*, are parasitic, infect over 2 billion people, and remain a significant health problem. Drug treatment is a key component of current helminth control programs and drug resistance has been identified in both human and veterinary helminths. The idea of searching for new solutions of treatment is therefore needed. The results obtained for a compound with a benzyl at the $N^2$ position ($bz^2m^7GMP$) encouraged me to extend these analyses to several other $N^2$ modified derivatives. A discovery of novel cap analogs that can specifically block parasite gene expression may be a first step in new drug development. Paper [**H8**] presents the synthesis and biological studies of $N^2$-substituted 7-methylguanosine 5'-monophosphates. This type of cap analogs has not been widely studied before, due to difficulties with the development of a good method to efficiently introduce substituents at the $N^2$ position of guanosine.

Over the years $N^2$-mono- or $N^2$,$N^2$-dimethylguanosine was obtained by Yamazaki method, which was modified and refined by myself. Unfortunately, after the year 2000 this method was no longer used as the starting substrate for the synthesis, AICA riboside (5-aminoimidazole-4-carboxamide), was not commercially available. These difficulties forced me to find a new method of preparation this crucial for TMG cap analogues synthesis compounds. Several methods are described in the literature for the synthesis of mono- and dimethyloguanosine but most of them are multi-step, time-consuming and leading to the desired product with low yields. Given that mono- or dimethylguanosine are not tested directly but are substrates for the synthesis of various cap analogs, the existing methods in their original form were not efficient enough. At that time I decided to focus on a method which allowed to obtain not only $N^2$, $N^2$-dimethylguanosine, but also other compounds modified at the $N^2$ position of guanosine by transformation of 2',3',5'-tri-*O*-acetyl-$O^6$-[2-(*p*-nitrophenyl)ethyl] guanosine to fully protected 2-fluoro inosine and nucleophilic substitution with dimethylamine. Described method was modified, since in its original form was not efficient enough (extremely low yields, difficulties with product purification). Extensive research on the isolation and purification of the Mitsunobu reaction product, diazotization and fluorination reactions and effective deprotection and purification of the final product led to development of a method that allows to obtain $N^2$,$N^2$-dimethylguanosine with very high yield (around 70% to the initial guanosine) together with significant shortening and simplifying the procedure. Developed strategy allowed to prepare several derivatives (Fig. 5) that possess $N^2$ substituents that vary in their size, steric branching and chemical nature (aliphatic or cyclic, aromatic or not, with introduced various substituents into benzene ring) [**H8**].

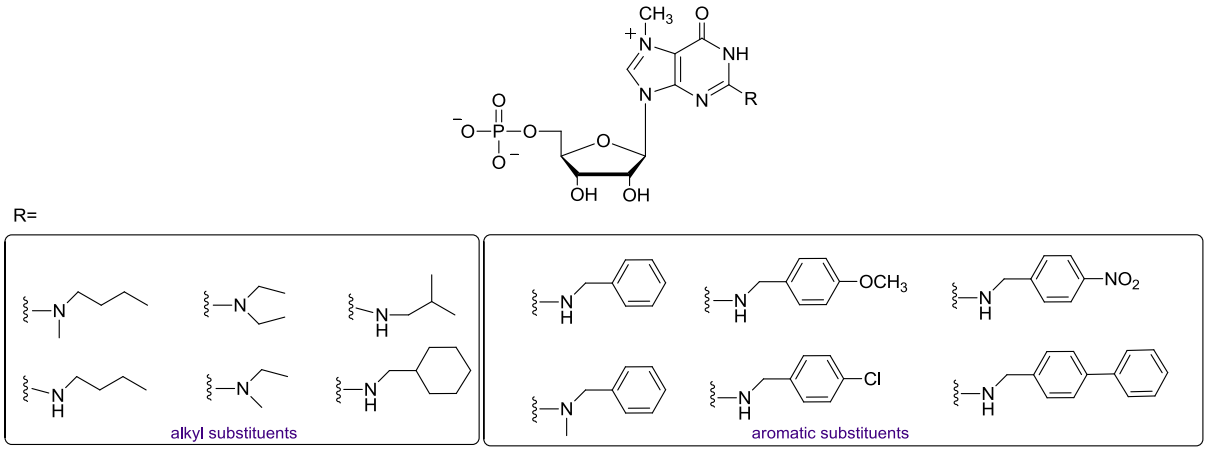



**Fig. 5** $N^2$-modified 7-methylguanosine-5'-monophosphates

Synthesized mononucleotide TMG cap analogues were tested in a parasitic nematode, *Ascaris suum*, cell-free system as translation inhibitors. Results indicated that any mono substitution at the $N^2$ position of guanine (alkyl–aliphatic, cyclic, or aryl) produced inhibitory compounds with $IC_{50}$ similar to $m^7GTP$. The strongest inhibitor $N^2$-*p*-metoxybenzyl-7-methylguanosine-5'-monophosphate is about 7 times stronger than $m^7GTP$. It is also important that the idea of exploring cap analogues that possess only one phosphate and typically have little inhibitory activity offers an opportunity to explore compounds that are not highly charged and can be good candidates for new drug development.

Efficient method for the preparation of $N^2$ substituted guanosine derivatives allowed me to design and prepare a set of mono- and dinucleotide cap analogs for studies on enzymes involved in the formation of TMG cap, guanine-N2 methyltransferases (Tgs, trimethylguanosine synthase) [**H2**]. Preliminary research conducted by Prof. Stewart Shumman's team (Memorial Sloan-Kettering Cancer Center, New York) for the newly discovered Tgs enzymes: human, yeast, viral (mimivirus) and protozoan lamblia, indicated that these enzymes form two distinct functional classes, depending on the final reaction product ($N^2$,7-dimethyl or $N^2$,$N^2$,7-trimethylated caps). In order to characterize the substrate specificity and to better understand mechanism of Tgs enzyme action, it was necessary to examine separately the first and the second reactions of methylation within the exocyclic amino group of guanosine. This studies were conducted using specially prepared cap analogs (5' mono-, di- and triphosphates and dinucleotides with tri- and tetraphosphate bridge) having one (position 7 or $N^2$), two (position 7 and $N^2$) or three (position 7, $N^2$ and $N^2$) methyl groups. In the case of dinucleotides I also prepared various analogues with modified second base within the cap structure. The results showed that human enzyme Tgs, performs successively two distinct methylation reactions, and other tested enzymes are only able to carry out the first methylation. The impact of the number of phosphates, substituent at the N7 position (none,

methyl, ethyl or benzyl), and other bases (G, A, C, 2'-deoxyG, 2'-O-metyloG) on enzymes activity was also explored. It was shown that the most important factor for methyltransferase activity is presence of the substituent at the N7 position.

## Summary

The most important achievements resulting from described publications are: design, synthesis and development of various methods of obtaining a number of new class of compounds such as:

- dinucleotide cap analogues functionalized within the second guanosine sugar moiety with levulinic acid allowing connection with various markers, probes and linkers that won't interfere the basic structural elements of the cap that are responsible for protein binding. The proposed method of functionalization is universal and can be used for other mono-, di- and oligo cap analogues [**H3**]
- affinity chromatography resins based on dinucleotide monomethyl and trimethyguanosine cap analogues, including these with increased resistance of the ligand to hydrolysis agaist DcpS enzymes [**H4**]
- oligonucleotides with a length of 22 bases with attached at the 5' end mono- or trimethylated cap structures [**H6**]
- dinucleotide mono- and trimethylated cap analogues labeled with $^{13}C$ and $^{14}C$ isotopes [**H7**]
- $N^2$ modified mononucleotide cap analogues (one or two substituents) [**H8**].

Furthermore, an efficient method for the preparation of $N^2$ substituted guanosine derivatives [**H8**] enabled to design and synthesize a set of dinucleotide cap analogues, having one, two or three methyl groups in various combinations (position N2 and N7) [**H2**] and all of compounds mentioned above, in which $N^2$,$N^2$,7-trimethylguanosine is present.

Prepared compounds are an indispensable tool for the research on cap binding proteins, including eIF4E isoforms with dual cap binding specificity. It should be mentioned that these analogues can be used to analyze protein-cap and protein-capped mRNA interactions using different biophysical techniques such as surface plasmon resonance, fluorescence quenching, electron paramagnetic resonance spectroscopy and magnetic resonance spectroscopy. Other important aspect is the ability to use obtained compounds as tools for searching of new proteins that recognize MMG and TMG caps, for monitoring chemical and enzymatic reactions involving the cap structure and using them in techniques of molecular biology and biochemistry (e.g. pull down assay, the inhibition of translation *in vitro*, synthesis *in vitro* of RNA transcripts). The most important results of already conducted studies using synthesized cap analogues are:

- understanding of kinetics of MMG cap binding by cap binding complex CBC. These data allowed to propose a model for this interaction [**H1**]
- identification of the important role of the spliced leader sequence and a TMG cap in the interaction with *A. suum* eIF4E-3 protein [**H5**]
- determination of the crystallographic structure of *A. suum* eIF4E-3 in complex with a TMG cap, quantitative characterization of their interactions and presentation of new data on the effect of steric hindrance associated with the presence of substituents within the $N^2$ exocyclic amino group on binding [**H5**]
- determination that all compounds which are $N^2$-monosubtituted analogues of $m^7$GMP are effective inhibitors of translation in *A. suum* and that the most effective translation inhibitor, 7 times stronger than $m^7$GTP, is a $N^2$-*p*-metoxybenzyl-7-methylguanosine-5'-monophosphate [**H8**]
- identification of different substrate specificity of $N^2$ guanine methyltransferases from a variety of organisms [**H2**].

These results bring us to learn about adaptation of nematode protein eIF4E interacting with MMG and TMG capped mRNAs during the initiation of translation, enzymatic mechanism of the formation of the TMG cap and interaction of CBC protein with the cap. The results also offer the opportunity to use this knowledge for therapeutic purposes such as anti-parasitic drugs. It should be noted that analogues described were thought to specific applications, but they may be used for other purposes to study the mechanisms of gene expression in eukaryotic organisms.

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## 6. Description of other scientific achievements

(*publications with the letter D are related to the list of publication after the Ph.D. degree on page 22, publication cited with the letter M are related to the list of publication before the Ph.D. degree on page 24)*

My main scientific interests are related to the 5' end of polymerase II transcripts and the role of the cap structure in various processes of gene expression. An important part of my research activities, outside the mainstream, described in previous paragraph, that is focused on the design and development of synthesis of new nucleotide compounds, are interdisciplinary studies in cooperation with both domestic and foreign centers, initiated in Poland and inspired by Prof. Edward Darżynkiewicz from Department of Biophysics, University of Warsaw.

At the beginning I was focused on the synthesis of relatively simple mono- and dinucleotide cap analogues, with different structure modifications. The

mononucleotide analogs possessed various number of phosphate groups, different substituents at the N7 position of guanine and varied amount of the base methyl groups [**M1**], [**M2**]. A set of dinucleotide cap analogs modified within the first or/and a second base moiety was also prepared [**M4**], [**M7**]. These compounds were subjected to a comprehensive physicochemical characteristics by determination of their dynamic conformational equilibrium [**M1**], [**M4**], [**M7**], [**M11**], and spectral properties of absorption (UV) and emission (fluorescence) [**M5**], [**M7**]. These analogues were also objects for biophysical studies of inter- and intramolecular interactions between the bases of the cap and indole ring of the tryptophan in the active center of eIF4E [**M6**], [**M8**], [**M11**], [**M14**], [**M16**] conducted by groups of Prof. Stolarski (Department of Biophysics, Faculty of Physics, Warsaw University) and Prof. Zbigniew Wieczorek (Department of Physics and Biophysics, Department of Food Science, University of Warmia and Mazury).

Synthesized cap analogs were also used in biological research [**M3**], [**M9**], [**M13**], [**M15**], [**M17**], and I will mention only the one in which my contribution was significant. Modified mRNA cap analogs were used to determine their ability to inhibit translation initiation in rabbit reticulocytes system *in vitro* [**M15**]. These studies were carried out in collaboration with Prof. Robert Rhoads from Louisiana State University Health Science Center in Shreveport, USA, and were carried out mostly by myself during my visit in his laboratory. Based on obtained data, earlier knowledge was verified and further correlation between the cap structure, and its activity in the process of translation initiation was identified. This research [M15] remains to this day the most comprehensive in terms of a catalog of tested compounds.

The second synthetic issue in my research was the creation of affinity resins for the isolation and purification of cap-binding proteins based on the idea that was used in commercially available matrix $m^7$GTP-Sepharose 4B with a 5'-$P^3$-4-aminophenyl-$P^1$-7-methylguanosine triphosphate (*p*-aminophenyl ester $m^7$GTP) as a ligand. Modified method for the preparation of such resins allowed me to prepare $m^7$GTP-Sepharose and $m_3^{2,2,7}$GTP-Sepharose [**M2**]. The former turned out to be more effective than commercial resin and for that reason it was used as a tool in [**M3**]. The most important from the practical point of view was $m_3^{2,2,7}$GTP-Sepharose as it enabled to carry out the search of potential new TMG cap-binding proteins. As it was mentioned in section 5.3, the TMG cap structure is present at the 5' end of most mRNA in nematodes. Studies on interaction of eIF4E with a regular MMG cap showed that this protein from higher eukaryotes strongly prefer MMG over TMG cap, suggesting that unknown cap-binding proteins that bind the hypermethylated form of the cap may exist in nematodes. Research in this direction was undertaken in collaboration with Prof. Robert Rhoads from Louisiana State University, Health Science Center in Shreveport, USA, and was carried out during my two visits to his laboratory. These studies [**M12**], [**M13**] led to overexpression and isolation of five *Caenorhabditis elegans* eIF4E isoforms and preliminary characterization of their binding affinities for both existing cap structures (MMG and TMG caps). The results showed that all isoforms, named IFE-1, IFE-2 etc. recognize the MMG cap and three

of them (IFE-1, IFE-2 and IFE-5) recognize and bind the TMG cap. It should be pointed out that it was the first described case showing the presence of eIF4E proteins that bind the hypermethylated version of the cap.

Detection of the first nematode *Caenorhabditis elegans* eIF4E isoforms that recognize MMG and TMG caps, and characterization of their varied specificity for both cap structures, directed my further research activities after obtaining a doctoral degree, to examine the phenomenon of dual specificity of nematode cap-binding proteins, and to synthesize various compounds that will be specially designed for such studies.

First undertaken research in collaboration with Prof. Robert Rhoads (LSUHSC, Shreveport) were aimed at structural aspects of differences in cap binding specificity of *C. elegans* isoforms. Based on a sequence analysis and a homologous modeling of IFE-3 (recognizes only MMG cap), and IFE-5 (recognize MMG and TMG cap), the sequence that may play a key role in binding a TMG cap was determined [**D1**]. The corresponding mutant proteins (amino acid sequence was changed to obtain a protein showing specificity only for MMG cap) were also tested for the recognition of both cap structures. Based on obtained results we presented a model that may explain the cause of dual specificity of various isoforms. It assumed the existence of steric hindrance in the binding pocket of IFE-3 isoform impeding the access of a $m_3^{2,2,7}$GTP (resulting from the narrower and shallower pockets than IFE-5) and the presence in both proteins an element that determine better binding of a $m^7$GTP than a $m_3^{2,2,7}$GTP (create a hydrogen bond with the exocyclic amino group of guanosine). In order to describe the quantitative affinity of *C. elegans* eIF4E isoforms to MMG and TMG caps I cooperated with biophysicists led by Prof. Ryszard Stolarski (Department of Biophysics, Institute of Experimental Physics, Department of Physics, University of Warsaw) and Prof. Zbigniew Wieczorek (Department of Physics and Biophysics, Department of Food Science, University of Warmia and Mazury). We chose three isoforms (IFE-3, -4, -5) as representatives of three classes that *C. elegans* eIF4E isoforms may be divided, taking into account: the primary structure, the binding specificity and the necessity of individual isoforms for the survival of nematodes. These proteins have been studied by spectroscopic methods and modeling based on homologous protein structure of mouse eIF4E in complex with $m^7$GDP [**D2**]. The qualitative data of specificity of two isoforms of mRNA cap structures obtained by affinity chromatography was confirmed by fluorescence quenching titration of protein by MMG and TMG cap analogs.

It is worth noting that a detection of different *C. elegans* eIF4E isoforms varying in the cap binding specificity, began searching for such kind proteins in other organisms. An example might be a detection of eIF4E-1 isoform in *Leishmania,* parasitic protozoa responsible for the transmission of dangerous diseases known as leishmaniasis [**D7**].

A key tool for all described research were the affinity resins with attached $m^7$GTP or $m_3^{2,2,7}$GTP. It is worth noting that these matrices have a very limited stability (even during storage at 4° C), and therefore they were prepared by me every time they were needed.

A substrate for TMG cap analogues synthesis, including affinity resins, is a $N^2$,$N^2$-dimethylguanosine. Modified and optimized procedure for the synthesis of $N^2$ substituted guanosine [**H8**] enabled me to prepare different mono and dinucleotide MMG and TMG cap analogs ($m_2^{2,7}$Guo, $m_3^{2,2,7}$Guo, $m_2^{2,7}$GMP, $et^2m^7$GMP, $m_3^{2,2,7}$GMP, $m_2^{2,7}$GDP, $m_3^{2,2,7}$GDP, $m_2^{2,7}$GTP, $m_3^{2,2,7}$GTP, $m_2^{2,7}$GpppG, $m_3^{2,2,7}$GpppG/A, $m_2^{2,7}$GppppG, $m_3^{2,2,7}$GppppG, $m^2bz^7$GppppG). These analogues were crucial for various biophysical and biochemical studies, for example:

- series of structurally modified mono and dinucleotide cap analogues was used to determine the association constants of murine factor eIF4E by team of biophysicists from the Department of Biophysics (Dr Anna Niedźwiecka and Prof. Ryszard Stolarski) using novel fluorometric, time-synchronized titration method [**D3**]. Obtained data allowed to determine the impact of individual structural elements of the cap-eIF4E complex and made it possible to estimate, based on the known crystallographic structure of eIF4E-$m^7$GDP, contributions to the Gibbs free energy of various non-covalent interactions between structural elements of the cap and protein, and to propose a two-stage model for cap binding .

- similar studies were carried out in solution with a human nuclear cap-binding complex (CBC, a cap-binding complex) [**D9**]. Determined association constants with structurally modified cap analogues revealed strong differences between eIF4E and CBC proteins. The data allowed to confirm a model of CBC binding to the cap, based on crystallographic structure.

- nucleoside, mono-, di- and triphosphate and dinucleotide analogues were used in systematic studies on the kinetics of the imidazolium ring-opening of 7-methylguanosine in alkaline solution [**D12**], allowing to define the effect of the individual structural elements for the decay rate.

- dinucleotide cap analogues were tested in collaboration with Prof. Robert Rhoads (LSU, Shreveport, USA) for their application in *in vitro* synthesis of capped mRNA transcripts with high translational efficiency [**D4**]. Such transcripts are widely used in the study of the mechanisms of translation, RNA splicing and transport in the cell. For all analogues ($m^7$GpppG used as reference) affinity for the initiation translation factor eIF4E, the ability of analogs to inhibit the *in vitro* translation, efficiency of analogues to incorporate into the transcripts during transcription *in vitro*, the level of incorporation of the analog to the transcript in the correct orientation and *in vitro* translation efficiency of mRNA with incorporated cap analogues, were determined. It was shown that: $N^2$-methyl substituent in the compounds $m_2^{2,7}$GpppG, $m_2^{2,7}$GppppG, $m^2bzl^7$GppppG has a positive effect for all five parameters, and the last of the aforementioned compound has the highest

reported so far efficiency of *in vitro* translation, it incorporates in the correct orientation, and is the most potent translation inhibitor. What is also important from the point of using TMG cap analogues to create transcripts useful in the study of U snRNA and mRNA translation of nematodes, this cap is incorporated into RNA much more efficiently than the MMG cap and mostly in the correct orientation.

- dinucleotide analogs of a MMG and a TMG cap were used as model substrates to study the specificity and kinetics of the cap hydrolysis by *C. elegans* DcpS enzyme [**D18**].

A significant part of my research activities after obtaining a doctoral degree was related to collaboration with Dr Richard Davis (Department of Biochemistry and Molecular Genetics, University of Colorado School of Medicine, USA), which deals with the mechanism of gene expression in nematodes. This type of organisms, which include model organism *Caenorhabditis elegans* and swine worm *Ascaris suum*, are characterized by *trans*-splicing of RNA as a part of gene expression. As a result a part of their mRNA (50-90%) acquires at the 5' end a spliced leader (SL) with an unusual for mRNA trimethylguanosine cap (TMG cap). A wide range of studies performed by Dr Davis is aimed at understanding the functional importance of *trans*-splicing and mechanistic adaptation of gene expression in relation to *trans*-spliced mRNAs possessing a TMG cap structure, with particular emphasis on the structure and function of proteins that recognize the cap structure in translation, transport, degradation, etc.. Our cooperation, which was based on experience and biochemical tools developed by Dr Davis (including RNA transfection by particle bombardment and cell-free translation and decay systems in Ascaris embryos, not available for *C. elegans*), and my experience in the field of synthetic cap analogues (especially, crucial in the context of this study, TMG cap analogues) and other chemical tools, (e.g. affinity resins) was therefore a natural extension of research on dual specificity eIF4E isoforms. The most important our achievements are:

- detection of enzymatic activity in *Ascaris suum* extract similar to that exhibited by the decapping enzymes (DcpS and Dcp1/Dcp2) from cells of higher eukaryotes and examination of their specificity for both cap structures (MMG and TMG) [**D5**]. Based on the prepared set of mono- and dinucleotide cap analogues, substrate specificity of *C. elegans* DcpS was identified. It was shown that it hydrolyzes dinucleotide analogues of MMG and TMG cap. Using an affinity resins: $m^7$GTP- and $m_3^{2,2,7}$GTP-Sepharose the binding affinity of enzymes were determined and compared with other proteins. These experiments confirmed that nematode DcpS, in contrast to the human protein recognizes both caps.

- preparation of RNA transcripts with different sequences, with or without spliced leader, capped with a MMG or TMG cap. Such transcripts allowed to:

- characterize an *A. suum* translational cell-free system developed in Dr Davis team (the first and the only for nematodes) [**D6**]. It was shown that this system reproduces all features of nematode translation *in vivo*. This enables to carry out a number of studies on *A. suum* translation (e.g. shown in our other paper).

- carry out studies on *in vivo* translation in *Ascaris suum* which allowed to determine impact of cap type (MMG or TMG), spliced leader sequence, polyadenylation of the 3' end and sequence between SL and a start codon on the *in vivo* translation efficiency. Although most of population of nematode mRNA contain a TMG cap at the 5' termini, its presence does not support translation as efficiently as MMG cap. Only synergistic effect of the SL and TMG cap leads to enhanced translation.

- point out a specific sequence within a spliced leader sequence which is required for the efficient translation (in *A. suum* cell free system) of TMG capped mRNAs [**D17**]. Studies using capped transcripts with various mutations within SL, indicate that the SL sequence forms a secondary loop structure, adjacent to the TMG cap, that seems to be crucial for efficient translation. This fact is related only to mRNAs with a TMG cap in nematodes.

■ use of affinity resins ($m^7$GTP-and $m_3^{2,2,7}$GTP-Sepharose), and MMG and TMG cap analogues to study translation of mRNA in nematodes. A recombinant *A. suum* eIF4E, similar as for *C. elegans,* was obtained and named eIF4E-3 [**D6**]. Based on various experiments using affinity chromatography with $m^7$GTP and $m_3^{2,2,7}$GTP-Sepharoses and appropriate mononucleotide competitors, it was found that eIF4E-3 binds both caps (MMG and TMG caps) and is involved in translation of both types of mRNAs.

■ carry out similar experiments for parasitic flatworm, *Schistosoma mansoni* [**D16**], which led to discovery of only one 4E isoform. Its sequence differs from human eIF4E compared to previously known *C. elegans* and *A. suum* isoforms. Based on affinity chromatography, it was found that protein binds both cap structures (MMG and TMG cap). Quantitative studies of protein affinity using fluorescence titration with different cap analogues revealed that the isoform binds to a MMG cap as strong as other proteins from higher eukaryotes, while a TMG cap is bound about 5 times weaker. Furthermore, prepared mono- and dinucleotide cap analogues were used to: determine thermodynamic parameters of protein interaction with MMG and TMG caps by isothermal titration calorimetry (ITC), to obtain crystals of protein in complex with MMG cap which enable to determine X-ray structure, and prepare cap-protein complexes which were examined by NMR. Obtained data showed that the ability of *S. mansoni* eIF4E to bind both caps is not due to fundamental differences in the mechanism of binding but is probably related to the internal flexibility and conformational changes of protein during cap binding.

Beyond the scope of the role of the 5' end of mRNA in the process of gene expression, in recent years I started a cooperation with Prof Ewa Bulska (Laboratory of Basic Aspects of Analytical Chemistry, University of Warsaw) for the research on a new measurement methodology for the identification of protein-metal connections in body fluids. This methodology involves separation of proteins by gel electrophoresis, and then metal detection by LA ICP MS technique (Laser Ablation Inductively Coupled Plasma Mass Spectrometry). The development of appropriate procedures for the separation of proteins by gel electrophoresis, which I am responsible for, in order to carry out the gel microsampling using LA ICP MS system, was a part of two PhD projects and will be presented in a paper soon. Obtained results indicate the possibility of using this methodology for monitoring platinum based chemotherapy.

**List of publications (apart from these related to the main achievement cited on page 3) after Ph.D. degree published in Journal Citation Reports and listed in Web of Knowledge:**


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D2. Stachelska A., Wieczorek Z., Ruszczyńska K., Stolarski R., Pietrzak M., Lamphear B.J., Rhoads R.E., Darżynkiewicz E. and Jankowska-Anyszka M. (2002): Interaction of three *Caenorhabditis elegans* isoforms of translation factor eIF4E with mono- and trimethylated mRNA 5' cap analogues, ***Acta Biochimica Polonica*** 49, 671-682.

D3. Niedźwiecka A., Marcotrigiano J., Stępiński J., Jankowska-Anyszka M., Wysłouch-Cieszyńska, Dadlez M., Gingras A-C., Mak P., Darżynkiewicz E., Sonenberg N., Burley S. and Stolarski R. (2002): Biophysical studies of eIF4E cap-binding protein: recognition of mRNA 5' cap structure and synthetic fragments of eIF4G and 4E-BP1 proteins, ***Journal of Molecular Biology*** 319, 615-635.

D4. Grudzien E., Stepinski J., Jankowska-Anyszka M., Stolarski R., Darzynkiewicz E., Rhoads R.E. (2004): Novel cap analogs for in vitro synthesis of mRNAs with high translational efficiency, ***RNA*** 10, 1479-1487.

D5. Cohen L.S., Mikhli C., Friedman C., Jankowska-Anyszka M., Stepinski J., Darzynkiewicz E., Davis R.E. (2004): Nematode M(7)GpppG and m(3)(2,2,7)GpppGdecapping: activities in Ascaris embryos and characterization of C-elegans scavenger DcpS, ***RNA*** 10, 1609-1624.

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D8. Worch R., Niedzwiecka A., Stepinski J., Jankowska-Anyszka M., Mazza C., Darzynkiewicz E., Cusack S., Stolarski R. (2005): Significance of the first transcribed nucleoside of capped RNA for ligand-induced folding of the cap-binding complex, ***Journal of Physics: Condensed Matter*** 17, S1495-S1503.

D9. Worch R., Niedzwiecka A., Stepinski J., Mazza C., Jankowska-Anyszka M., Darzynkiewicz E., Cusack S., Stolarski R. (2005): Specificity of recognition of mRNA 5 ' cap by human nuclear cap-binding complex, ***RNA*** 11, 1355-1363.

D10. Worch R., Stepinski J., Niedzwiecka A., Jankowska-Anyszka M., Mazza C., Cusack S., Stolarski R., Darzynkiewicz E. (2005): Novel way of capping mRNA trimer and studies of its interaction with human nuclear cap-binding complex, ***Nucleosides Nucleotides & Nucleic Acids*** 24, (5-7): 1131-1134.

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