Dynamics of miRNA biogenesis and nuclear transport

Aneesh Kotipalli¹, Ravikumar Gutti¹, Chanchal K Mitra¹,*

¹Department of Biochemistry, School of Life Sciences, University of Hyderabad, Hyderabad 500046, India, http://www.uohyd.ac.in/

Summary
MicroRNAs (miRNAs) are short noncoding RNA sequences ~22 nucleotides in length that play an important role in gene regulation-transcription and translation. The processing of these miRNAs takes place in both the nucleus and the cytoplasm while the final maturation occurs in the cytoplasm. Some mature miRNAs with nuclear localisation signals (NLS) are transported back to the nucleus and some remain in the cytoplasm. The functional roles of these miRNAs are seen in both the nucleus and the cytoplasm. In the nucleus, miRNAs regulate gene expression by binding to the targeted promoter sequences and affect either the transcriptional gene silencing (TGS) or transcriptional gene activation (TGA). In the cytoplasm, targeted mRNAs are translationally repressed or cleaved based on the complementarity between the two sequences at the seed region of miRNA and mRNA. The selective transport of mature miRNAs to the nucleus follows the classical nuclear import mechanism. The classical nuclear import mechanism is a highly regulated process, involving exportins and importins. The nuclear pore complex (NPC) regulates all these transport events like a gate keeper. The half-life of miRNAs is rather low, so within a short time miRNAs perform their function. Temporal studies of miRNA biogenesis are, therefore, useful. We have carried out simulation studies for important miRNA biogenesis steps and also classical nuclear import mechanism using ordinary differential equation (ODE) solver in the Octave software.

1 Introduction

1.1 Micro RNA biogenesis
MicroRNAs (miRNA) are short, single stranded, non-coding RNA molecules; ~21-24 nucleotides in length and are reported to play vital roles in gene regulation [1]. They were first identified in the nematode Caenorhabditis elegans as a postembryonic developmental event regulation [2]. miRBase (http://www.mirbase.org/; a large repository of microRNA sequence information) today has more than 28,000 microRNAs identified in both vertebrates and invertebrates [3]. Under the Homo sapiens category, 2588 mature, highly curated, microRNA sequences and some of their known mRNA targets are available in miRBase. miRNAs regulate many different cellular processes like differentiation, proliferation, maturation and development and may be involved in pathogen-host communication [4], [5]. MicroRNAs are ubiquitous in the genome, some are from coding genes, some are from intron regions as non-coding transcripts, and some are from gene promoter regions, as primary transcripts by the cellular machinery [6]. The primary transcript forms a hairpin (stem loop) structure, with some bulges due to base-pair mismatch, a polyA tail and a 5'-cap end. They are commonly referred to as primary miRNAs (pri-miRNA). Pri-miRNAs are processed into precursor
miRNA (pre-miRNA) by the action of Drosha-DGCR8 (DiGeorge syndrome critical region8) complex by removing polyA tail and 5’-cap end within the nucleus. Drosha is a member of the RNase III enzyme family and plays a major role in cleaving the 5’-cap and polyA tails. DGCR8 helps in the identification of cleavage site within the pri-miRNA structure. Drosha contains two main domains: RIIIDa cleaves the 3’ strand and RIIIDb which cleaves the 5’ strand adjacent to the hairpin. Drosha-DGCR8 complex is also known as the microprocessor complex [7], [8].

After the formation of pre-miRNAs in the nucleus they are transported to the cytoplasm with the help of exportin-5 in presence of Ran-GTP (a Ras related nuclear protein) [9]. Exportin-5 is a protein from the karyopherin family and is involved in the nuclear transport of structured RNAs such as tRNAs, human Y1RNA and adenovirus VA1RNA all of which have a 3’ overhang structure. The Exportin-5 and Ran-GTP complex recognises the 2-nucleotide 3’ overhang structure of the pre-miRNA. So it indicates the recognition of pre-miRNA by Exportin-5 is mostly by structure of pre-miRNA rather than the sequence of the RNA. The exportin-5 and Ran-GTP complexes are playing a major role in the transport of this trimeric complex (Pre-miRNA, Exportin-5 and Ran-GTP) across the nuclear membrane through the nuclear pore complex [10]. It is cumbersome to experimentally detect pre-miRNAs and their effects and, therefore, computational studies have become indispensable [11], [12].

In the cytoplasm a second set of processing steps takes place with Dicer (an RNase III enzyme) which with the help of TRBP (Trans activation response RNA binding protein) forms a double stranded miRNA of ~21-24 nucleotide length. Dicer is a ~218kDa protein with a central PAZ (Piwi-Argonaute-zwille) domain which is involved in the interaction with the pre-miRNA [13]. Upon completion of the Dicer action, the pre-miRNA exists as a Double stranded RNA duplex with ~21-24 nucleotides in length. One of the two strands is degraded, and is known as the passenger strand. The other strand is the mature RNA or guide RNA. The selection of mature or guide strand is based on the thermodynamic stability. The strand with less stability is selected by the RISC (RNA induced silencing complex) [14]. The mature strand is loaded into RISC by RLC (RISC loading complex). RISC consists mainly of Dicer, TRBP, AGO2 (Argonaute protein 2) and PACT (Kinase R-activating protein) [15]. The mature miRNA can be carried back to the nucleus via the nuclear pore complex using a very similar mechanism.

The mature miRNA within the RISC complex shows its regulatory role in the cytoplasm as well as in the nucleus [16]. In the cytoplasm targeted mRNA 3’UTRs with complementary sequence show translational repression, sometimes multiple miRNAs target a single mRNA and vice versa [17], [18].

In the nucleus also miRNAs show their role in gene regulation, mature miRNAs with a nuclear localisation signal (AGUGUU) at the 3’ terminus will be destined to the nucleus [19]. Importin-8 is the karyopherin beta family protein which helps in the nuclear import of mature miRNAs further aided by argonaute proteins [20]. These nuclear miRNAs show their action at gene promoter regions with the help of argonaute proteins and also recruit the epigenetic modifier proteins such as (CBX3, TIF1B, SUV39H1, EHMT2) at promoter region and lead to transcriptional gene silencing (TGS) or transcriptional gene activation (TGA) [21–24]. A well-studied example is the MicroRNA-373 targeted promoter E-cadherin and CSDC2 induces the transcriptional gene activation [25]. Figure 1 summarizes the above steps schematically.

MicroRNAs regulate cell dynamics at different stages (time) of cell development, and they have relatively short half-life. They are gene products and are produced on demand and show their action and are then degraded within a short period of time. This makes it difficult to investigate turnover experimentally (similar to pre-miRNAs). Yet, it is important to understand their effects on various cellular metabolites as a function of miRNA lifecycle. In the present report we have simulated the miRNA biogenesis and nuclear transport mechanism to study its dynamics.
Figure 1: MicroRNA biogenesis and nuclear transport and gene regulation. Schematic diagram shows the series of reactions involved in the formation of mature miRNA and the role of miRNA in gene regulation [6-8, 10, 13, 18, 23-24, 28]. Only part of this series was considered for simulation.

1.2 Nuclear Transport

The Nuclear Pore Complex (NPC) is one of the major complex structures in the cell and is located within the nuclear membrane. It acts as a molecular gatekeeper between the nucleus and the cytoplasm; it allows the transport of various biological molecules (proteins, transcription factors, RNAs-mRNA, t-RNA, miRNA, ribosomal subunits etc.) through the NPC in a highly selective manner. Molecules with a size less than 40kDa (like ions, small molecules, small metabolites) diffuse passively in and out, whereas molecules with a size greater than 40kDa (like proteins, RNAs etc.) require a complex process with the involvement of exportins or importins to allow the facilitated diffusion of macromolecules [26]. The translocation in the NPC is a rapid process and occurs at a rate of ~1000 translocations per second [27]. There are several distinct exportins and importins reported and each has a specific function, transporting a unique cargo. For example exportin-5 is involved in the transport of pre-miRNAs [28], [29] from the nucleus to the cytoplasm and importin-8 is involved in the import of mature miRNA with the help of AGO2 into the nucleus [20], [30].

The number of NPC per cell differs from cell to cell, and it also depends on the cell size. Actively dividing cells have a larger number of NPC per cell. It has been reported that Hela cell line nuclear membrane contains ~3000 NPCs per cell [31]. The nuclear pore complex
(NPC) shows eight fold rotational symmetry. Each NPC consists of ~30 different nucleoporins (nups) which are the building blocks of the NPC and ~500 nups will form a single NPC [32]. The catalogue of each NUP is available and each NUP has its own functional role in the NPC architecture [33]. The FG (Phenylalanine-glycine) nups are mainly located in the centre of the NPC and are the main key players in the selective passage of molecular cargo from NPC in a bidirectional way. There are ~200 FG nups per NPC and they are mainly located in the centre of the pore [34], [35].

The Ran (Ras related nuclear protein) cycle in the cell is mostly regulated by the Ran gradient throughout the nuclear membrane [36]. RanGTP concentration is higher in the nucleus and is regulated by RanGEF. The RanGDP concentration is higher in the cytoplasm and is regulated by RanGAP. This Ran cycle is maintained by NTF2 (Nucleotide transport factor 2), and is involved in the loading and unloading of export and import cargo [36–39]. NTF2 exports RanGDP from the cytosol to the nucleus. RanGDP is converted to RanGTP in the nucleus in the presence of RanGEF. After the release of RanGDP in the nucleus, NTF2 goes back to the cytoplasm and is ready for another round of RanGDP transport. The mechanism behind the facilitated transport of NTF2 through NPC is by interaction with FG nups present at the core of the NPC [40].

The selective transport through NPC depends on the NLS (Nuclear localization signal) and NES (Nuclear Export signal) signals present on the cargo molecule [41], [42]. If NLS is loaded with a cargo molecule it moves to the nucleus through NPC [42–46]. If NES is loaded with cargo molecule then it moves to the cytoplasm through NPC. The molecular recognition of these export and import signals is provided by a family of karyopherins, importins, exportins and transporters [47]. Many different NLS sequences have been identified and the most studied are classical NLSs [48]. The classical NLSs (cNLS) is mostly composed of lysine (K) and arginine (R) amino acids. A well-studied example in this case is the SV40 large T-antigen with cNLS consisting of the PKKKRKV sequence. In cNLS, the cargo with NLS is primarily identified by its adaptor protein: importin-alpha and then bound to importin-beta forms a trimeric complex and transported to the nucleus through FG nup interaction at core of the NPC. Another type of NLS sequence identified is a Proline- Tyrosine NLS (PY-NLS) rich sequence, an example for PY-NLS is hnRNP A1 with an NLS sequence of SSNFGPMKGGNRFFRSSGPY. The main characteristic feature of PY-NLS sequences are recognized by Kapβ transport receptor and no adaptor proteins are required as they are in the case of cNLS adaptor proteins [47].

The following steps are involved in the classical NLS import process. Importin alpha recognizes the cargo with NLS sequences and this complex is recognized by importin beta which is a potential partner for importin alpha [48]. Then this trimeric complex is moved to the NPC in a facilitated diffusion manner. The FG nups interaction with importin beta allows this trimeric complex to then cross the nuclear membrane. Within the nucleus the release of cargo takes place with the help of RanGTP [49]. RanGTP binds to the allosteric site present on importin beta inducing a conformational change and consequently the release of the importin alpha–cargo-NLS complex [49]. Importin alpha binds with cse1 (chromosome segregation protein)/CAS (cellular apoptosis susceptibility) in vertebrates and release its cargo [50]. Again importin alpha-cse1/CAS binds to RanGTP and exports it back to the cytoplasm enabling another round of nuclear import [51], [52]. Importin Beta binds another molecule of RanGTP present in the nucleus and exports it back to the cytoplasm allowing for the next round of import [36]. The above steps are shown schematically in Figure 2.
Figure 2: Classical nuclear import mechanism and Ran cycle. Mature miRNA (NLS-cargo) is transported across the nuclear membrane via the nuclear pore complex (NPC) assisted by several factors like importins, exportins and RanGTP. Drawn based on the information from the references [36, 39, 40, 48-52] and used in the present simulation.

2 Methodology

The Michaelis-Menten (MM) equation is generally used to study enzyme kinetics [53]. In the present paper we extended the applicability of the MM equation to the simulation of miRNA biogenesis and nuclear transport. In general, an enzyme binds with its substrate to form the enzyme-substrate complex which subsequently decomposes to give the product(s). See the equation below

\[ E + S \rightleftharpoons k_2 k_1 ES \rightarrow E + P \]

The standard derivation assumes steady state conditions for the ES, the enzyme substrate complex. This means \( \frac{d[ES]}{dt} = 0 \). This means we can define a new constant

\[ K_m = \frac{(k_{-2} + k_2)}{k_1} \] and \( V_{\text{max}} = k_2 \cdot [E]_0 \)

The general formula for the MM equation is below:

\[ V = \frac{V_{\text{max}} [S]}{K_m + [S]} \]
Where \( V \) is initial velocity of the reaction, \( K_m \) is the MM constant (dissociation constant of the enzyme substrate complex), and \( [S] \) is the substrate concentration. The original three kinetic constants have been now reduced to two parameters. We rearrange the above equation to

\[
\frac{V}{V_{max}} = \frac{[S]/K_m}{1 + [S]/K_m}
\]

\( K_m \) depends on the stability of the enzyme-substrate binding (\( k_1 \) and \( k_{-1} \)). These parameters are sometimes not known for the majority of enzymes and their respective substrates.

\( [S] \) and \( K_m \) have the same dimensionality; hence \( [S]/K_m \) is a dimensionless number only. Similarly \( V/V_{max} \) is a unit-less number. As a result of scaling, time also becomes a dimensionless quantity [54].

The substrate concentrations, although reported in arbitrary units, are in reality in units of \( K_m \) of the respective enzymes. In the present case, we use \( [S]/K_m \) as the dimensionless quantity representing the concentration and explicit knowledge of \( K_m \) is not needed. However, if we need the actual concentration for a given component we need to know the specific \( K_m \) value. In reality, \( K_m \) values for a particular enzyme that were reported by different authors vary widely and eliminating \( K_m \) in this way appears to be an advantage.

The above equations are used for single substrate, but many biochemical reactions are multi substrate reactions. So we extended the equation for multi substrate using the formula below.

\[
\frac{V}{V_{max}} = \frac{[S_1]}{1 + [S_1]} \cdot \frac{[S_2]}{1 + [S_2]} \cdot \frac{[S_3]}{1 + [S_3]}
\]

In analogy to the rate equations (where the rate is proportional to the products of the reactants), we can safely approximate the overall rate as indicated above, where the individual concentrations have been replaced as \( \frac{[S]}{1 + [S]} \). Inhibitors and regulators of enzymes can be modelled in a similar way.

By using the above approximations we simulated the microRNA biogenesis and nuclear transport using ordinary differential equations (ODE) in GNU-Octave software version 4.0.3 (https://www.gnu.org/software/octave/). Octave is a high level interpreted tool generally used for numerical calculations to solve solutions related to both linear and non-linear problems. In the present work we used lsode (Livermore Solver for Ordinary Differential Equations) method in octave for simulations [55]. In this case, the linear first order differential equations are written in the form: \( f(x,y,y',y'',\ldots,y^n) \) where \( y^n = d^ny/dx^n \). Where \( y \) and \( y' \) are vectors usually supplied as a function and \( n \) is called the order of the equation.

Computer simulation of the kinetics of the metabolic pathways is important because it shows us the underlying dynamics of the various interactions present between different metabolites as mediated by enzymes. The problem is further complicated by the fact that various enzymes are regulated by other metabolites. For a very simple reaction pathway the final steady state concentrations of the different metabolites may be possible to predict by careful observation but the problem becomes very difficult in a real life scenario. Within a cell there are different compartments reserved for different sets of metabolic processes and the products and reactants between compartments interact via diffusion only.
3 Materials

The sequence of reactions undertaken in this study are listed in Table 1 below. The details of the enzymes are not mentioned by name in some of the reactions. The 29 reactions listed in Table 1 are simulated using the script (supplementary information). The script can be directly used in octave software (m-file). This m-file will also work in MATLAB with minor changes.

Table 1: Metabolic reactions used in simulation

| S. No | Metabolic reactions (series of reactions considered for simulation in the present study) | Reference |
|-------|----------------------------------------------------------------------------------------|-----------|
| 1.    | Drosha + DGCR8 ------> Complex-1                                                       | 7         |
| 2.    | Complex-1 + pri-miRNA ------> Pre-miRNA1-n+DGCR8+Drosha                               | 8         |
| 3.    | Exportin-5-n + RanGTP-n ------> Complex-2                                               | 10        |
| 4.    | Complex-2 + pre-miRNA1-n ------> Complex-3n                                              | 10        |
| 5.    | Complex-3n ----NPC------> Complex-3c                                                     | 10        |
| 6.    | Complex-3c ------->Pre-miRNA1c+RanGTP-c+Exportin-5-c                                    | 10        |
| 7.    | Dicer + TRBP ------> Complex-4                                                         | 13        |
| 8.    | Complex-4 + Pre-miRNA1c ------> ds-miRNA + TRBP + Dicer                                | 13        |
| 9.    | ds-miRNA + Ago2 ------> Mature miRNA + Ago2                                            | 13        |
| 10.   | Mature-miRNA+Ago2+ Complex-4 + PACT ------> RISC                                      | 9         |
| 11.   | RISC-NLS-Cargo-c +Importin-alpha-c ------> Complex-5c                                 | 48        |
| 12.   | Complex-5c + Importin-Beta-c ------> Complex-6c                                       | 48        |
| 13.   | Complex-6c ----NPC-FG-NUP----> Complex-6n                                              | 48        |
| 14.   | Complex-6n+RanGTP-n ------> Complex-5n + Importin-beta-n + RanGTP-n                   | 49        |
| 15.   | Complex-5n + CAS/cse1-n ------> NLS-Cargo-n + Importin-alpha-n + CAS/cse1n             | 50        |
| 16.   | Importin-alpha-n + CAS/cse1-n + RanGTP-n ------> complex-7n                             | 51        |
| 17.   | complex-7n ----NPC----> complex7c                                                      | 52        |
| 18.   | complex-7c ------->Importin-alpha-c +CAS/cse1-c+RanGTP-c                               | 52        |
| 19.   | Importin-beta-n + RanGTP-n ------> complex-8n                                          | 36        |
| 20.   | complex-8n ------NPC------> complex-8c                                                 | 36        |
| 21.   | complex-8c ------->Importin-beta-c + RanGTP-c                                          | 36        |
| 22.   | NTF2-c + RanGDP-c ------> complex-9c                                                   | 40        |
| 23.   | complex-9c ----NPC----> complex-9n                                                     | 40        |
| 24.   | complex-9n -------> NTF2-n + RanGDP-n                                                  | 40        |
| 25.   | NTF2-n ------NPC------> NTF2-c                                                         | 39        |
| 26.   | RanGDP-n ---- RanGAP ------> RanGTP-n                                                  | 40        |
| 27.   | RanGTP-c ---- RanGAP ------> RanGTP-c                                                  | 40        |
| 28.   | CAS/cse1-c ----NPC------> CAS/cse1-n                                                   | 51        |
| 29.   | Exportin-5-c ----NPC------> Exportin-5-n                                               | 28        |
4 Results

The metabolite concentrations (arbitrary units) seem to decay in a predictable manner during the time course of the simulation (Figure 3). The results obviously depend on the initial concentrations of the metabolites (They are seen in the script). The metabolite concentrations are dimensionless quantities expressed in terms of the respective Km values. In the same way the time axis (x-axis) is also dimensionless. This can be compared to experimental values only after proper scaling of the X and Y axes. However the relative changes are important and we can see clearly how the concentrations of various metabolites change as the reaction progress (Figure 4).

In Table 2 we have compiled the physical concentrations of some of the metabolites. We could not locate Km values for most of the enzymes/carriers. Drosha and dicer are ribonuclease III family members and are likely to have similar Km values. We note that dimensionless concentration of 0.5 for pri-miRNA translates to an effective concentration of 3nM. Assuming a typical cell volume of 1e-18 m³ (= 1e-15L), 1M solution has (1e-15 *6e23=6e8) 6e8 molecules present in the cell. 1nM concentration effectively translates to 1or 2 molecules.

The major observation is the miRNA ends up in the RISC complex and stays there until used up. The present simulation does not go beyond the formation and transport of the RISC complex.

| S. NO | Metabolite   | Enzyme/carriers | Km (nM) | Initial concentration (Dimensionless) | Final concentration at 200 Time/arbitrary units (Dimensionless) | Initial concentration (Km conc) | Final concentration (Km con) | Reference |
|-------|--------------|-----------------|---------|--------------------------------------|---------------------------------------------------------------|---------------------------------|---------------------------------|-----------|
| 1.    | Pri-miRNA    | Drosha          | 6       | 0.5                                  | 1.005e-04                                                    | 3 nM                            | 0.6 pM                          | [56]      |
| 2.    | Pre-miRNA1n  | Exportin-5      | Not found| 0.0                                  | 3.84e-04                                                     | -                               | -                               | -         |
| 3.    | Pre-miRNA1c  | Dicer           | 6       | 0.0                                  | 3.52e-04                                                     | 0.0                             | 2 pM                            | [56]      |
| 4.    | DS-miRNA     | AGO2            | Not found| 0.0                                  | 2.32e-04                                                     | -                               | -                               | -         |
| 5.    | Mature-miRNA | RISC            | Not found| 0.0                                  | 0.13                                                         | -                               | -                               | -         |

Km values for most of the above substrates /ligands could not be located in the literature. Brenda (http://www.brenda-enzymes.org) lists both dicer and drosha as ribonuclease III. We have therefore taken both dicer and drosha to have the same Km.

The simulation results are summarized graphically in the three graphs below. Because many metabolite concentration profiles plotted on a single graph makes it difficult to appreciate various features, we have plotted at most six metabolites in one graph. In figure 3 we show the concentration profiles for pri-miRNA, ds-miRNA, mature miRNA and RISC complex. Only pri-miRNA has been given an initial concentration of 0.5 units. Mature miRNA reaches a concentration of ~0.2 units around 100 time units but slowly decreases to ~0.08 units around 400 time units. The rest of the mature miRNA is exported to (not shown in this figure) nucleus as RISC-complex as NLS-cargo. Figure 4 shows the import of NLS-cargo via nuclear pore complex (NPC) with help of importin factors. NLS-cargo concentration can be seen to increase steadily ~0.42 units at 400 time units. The scripts can be user modified to see special
features or details of other metabolites. We have selectively shown relevant metabolites for the three sets of plots given below. The initial concentrations are important in a relative sense because if we reduce pri-miRNA concentration to 0.25 (say) other concentrations will be also reduced in a predictable manner. Finally dynamics of the pri-miRNA, mature miRNA and NLS-cargo are seen in figure 5.

Figure 3: miRNA biogenesis simulations. Only six metabolite concentration profiles are shown. Only pri-miRNA has a non-zero initial concentration in the simulation.

Figure 4: Classical Nuclear import simulations. In this simulation importin initial concentrations are non-zero and the result is almost quantitative transport of NLS-Cargo-n.

Figure 5: miRNA biogenesis and nuclear re-export. This graph is essentially a combination of the previous two graphs. The pri-miRNA (dark blue curve) is processed and mostly transferred as NLS-Cargo-n (across the nuclear membrane to the other side).

5 Discussion

In Figure 3 we see the processes that are responsible for microRNA biogenesis. This simulation includes pri-miRNAs as the starting metabolite. Note that the transcription kinetics have not been included in the present simulation. The pri-miRNA is then converted to pre-miRNA by the action of the Drosha-DGCR8 complex (see script in supplementary file for details). We see the rapid conversion of pri-miRNA into pre-miRNA (in the graph labelled as pre-miRNA1n; this occurs in the nucleus). This pre-miRNA is then transported to the cytoplasm rapidly with the help of Exportin-5 and RanGTP through the nuclear pore complex (NPC). The cytoplasmic components (the remaining four curves) show broad peaks that decay
slowly. Part of the final product (the mature miRNA as an important RISC component) moves back to the nucleus via the NPC and the importin complex. The nuclear component of the mature miRNA is not shown in Figure 3.

In Figure 4, we simulated the classical nuclear import mechanism. This is the simulation seen in Figure 3 but we plot different metabolites for reasons of visual clarity. It is convenient to study this plot together with Figure 3 because the conditions are the same. This is true also for Figure 5 where we provide relevant sets of metabolites. Figure 4 presents the metabolites involved in the transfer of NLS (Nuclear localization signal) cargo containing mature miRNA as RISC complex. The initial delay in the reactions can be understood when seen together with Figure 3. The concentrations of importin beta (green curve for cytosolic component and blue curve for the nuclear component) undergoes changes as they bind to the RISC complex and importin alpha (both cytosolic, blue color in the graph, and nuclear, pink color in the graph, concentrations are considered independently). As the reaction progresses the NLS cargo containing mature miRNA as RISC complex increases steadily, as expected.

In Figure 5, we have a combination graph that shows relevant metabolites. The blue curve represents the pri-miRNA (same as the blue curve in Figure 3) and the yellow curve represents the NLS-cargo as mature miRNA with RISC complex (same as the yellow curve in Figure 4).

The simulations are depended on the initial values of the various components in the reaction. The results might be different if different initial values were chosen. However realistic concentrations for most of the components are not available in the literature. Therefore the simulation remains a theoretical model waiting to be refined with experimentally results.

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