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Thesis Changes Log

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PhD Program: Materials Science and Engineering

Title of Thesis: Fluorescent Photoconvertible Polymer Microcapsules for Cell Labeling and Tracking
Supervisor: Prof. Gleb Sukhorukov

The thesis document includes the following changes in answer to the external review process.

The changes are mentioned in response to Reviewer's comments. I am grateful to the Reviewers for their valuable comments. I believe they helped me to make the thesis more complete and beneficial to the readers.

Professor Li Shang

Comment 1:
The role of PVA gel in enhancing the property of photoconvertible microcapsules has been mainly attributed to the increase of viscosity during the hydrothermal synthesis in the discussion of Chapter 4. How about other polymers with similar property? What if replace PVA with other substances that can increase the viscosity of the solution?
Change 1:
PVA is a water-soluble, non-toxic, biocompatible, and biodegradable polymer [252], which is important for the production of materials for use in biological systems. It is also thermally stable since it decomposes around 220 ◦C [253], which makes PVA gel a perfect candidate as a medium for capsule production.
In addition to PVA, other polymers can potentially be used as a medium for capsule synthesis. For example, poly(ethylene oxide) (PEO), which is water-soluble, biocompatible, and biodegradable, and it decomposes around 320 ◦C [257], demonstrating high thermal stability. Depending on the PEO molecular weight, similar to the PVA viscosities could be obtained [258]. Another option for capsule treatment medium might be water-soluble and biocompatible polyvinylpyrrolidone (PVP), which decomposes around 380 ◦C [259]. In addition, polyacrylic acid (PAA) is also water-soluble and biocompatible, and it starts to decompose between 230 and 300 ◦C [260]. Therefore, it can be potentially used for capsule thermal treatment. Nevertheless, concentrations of polymer gels should be carefully adjusted to obtain appropriate viscosity
This text has been added in Chapter 5 (Section 4.2).

Comment 2:
Two different types of photoconvertible microcapsules have been prepared: one is prepared by hydrothermal treatment with dyes physically encapsulated (Chapter 4); one is prepared by chemically modifying dyes to polymers without hydrothermal treatment (Chapter 5). Then, why choosing the materials prepared by the first strategy for cell imaging experiments in Chapter 6? What will be their advantage compared with these prepared by the second strategy?
Change 2:
Both types of capsules prepared with thermal (Chapter 4) and non-thermal treatment (Chapter 5) are expected to be applicable for cell tracking. In this chapter, capsules prepared with thermal treatment were

chosen for cell experiments for several reasons. The decrease in size during synthesis leads to the increase in shell thickness and stiffness. Therefore, capsules can be observed as separate bright "beads" in the cells that do not deform and do not collapse with each other. A smaller diameter of capsules is also beneficial for uptake by smaller cells (for example, fibroblasts [299]). At the same time, regular LbL capsules prepared without thermal treatment can deform due to their thin soft shell [300]. This might lead to their adhesion to each other and complicate the distinction of separate capsules in the cell for effective labeling. Note that such LbL capsules potentially could be used for drug delivery since no heating is required for their production, which is crucial for the drug. Moreover, this synthesis technique is suitable for the dyes that do not withstand thermal treatment. Nevertheless, for the goals of this research, thermally treated capsules were chosen for labeling experiments, taking into account their discussed advantages over LbL capsules.
This text has been added in Chapter 6 (Introduction).

Comment 3
In the cell experiments, the results showed that no microcapsules were observed to transfer from mMSCs to EPNT-5. Cells are known to have an exocytosis machinery for foreign substances. So, it is interesting to know why these microcapsules can retain inside the cells without being excreted for such a long time.
Change 3:
Cells are known to have exocytosis machinery, when after uptake, digestion, and nutrient absorption, cells remove undigested particles to the outside. The duration of capsule retention inside the cells depends on the cell line and polymers used for the production of the capsules. For example, microcapsules based on biodegradable PArg and DS were broken down by macrophages within 24 h [241]. At the same time, the capsules prepared with non-biodegradable polymers PAH and PSS polymers can be observed in cells for several days [331]. It was demonstrated in a work by Luo et al., where capsules remained inside C2C12 cells for 34 days [249].
This text has been added in Chapter 6 (Section 6.1.3.1 hMSCs).

Comment 4:
How was the number of microcapsules determined in the study? Experimental parameter for performing the photobleaching study in section 5.7 is also missed. Relevant details should be given clearly.
Change 4:
The concentration of capsules was counted using a hemocytometer (Goryaev chamber).
This text was added in the section 3.2.1.2. (Hydrothermal synthesis).
The photobleaching parameters were added as a separate subsection "Capsules photostability measurements" in section 3.3.4.2 (CLSM studies of fluorescent polymer microcapsules):
The photostability of the capsules containing the dyes was evaluated by collecting a time series of 15 scans at standard settings usually used for obtaining high quality images of the cells. The settings included a 1024 × 1024 pixel spatial resolution, a 100 Hz scanning speed, and the following LPDs: 46 mW/cm^2 for Fl, AY, AO, and TR; 6 kW/cm2 for RhB and Cy3.5; 18 kW/cm^2 for Rh6G and PyB;
and 9 kW/cm^2 for Cy5.5.

Dr. Andrei Sapelkin

Comment 1:
There are some very minor English language idiosyncrasies (ad a few typos) that I would like to see corrected, for example in the abstract, "Optical labeling of cells is usually performed via genetic modification for the following generation of photoconvertible proteins" should be changed to "Optical labelling of cells is usually performed via genetic modification to follow generation of photoconvertible proteins"; in page 7 "Moscow State University, Physical Department" has to be changed to "Moscow State University, Physics Department" etc. Please check again for English language throughout.
Change 1:
All mentioned by Dr. Andrei Sapelkin corrections have been included in the text of the thesis. The English language was checked and corrected throughout the whole text.

Comment 2:

In the Abstract there are very good introductory remarks, problem statement and brief description of the methodology to address the problem. However, there is no information about the main findings: e.g. We found that …" or "we show that …". Abstract should essentially be a shortened version of the conclusions summarising briefly the main results of the work.
Change 2:
Part of the Abstract was modified to list the main findings:
We demonstrated that the treatment of capsules in PVA gel with fluorescent dyes increased the yield of capsules from one synthesis by at least 250 times in comparison to the synthesis in water. Thermally treated capsules containing RhB and Rh6G were found to be photostable and exhibited photoconversion properties. We also found that capsules prepared without thermal treatment and containing Cy3.5, Cy5.5, and RITC demonstrated profound photoconversion properties. In cell experiments, it was demonstrated that capsules remained inside the cells for up to four days of tracking, preserving a bright fluorescence signal and allowing tracking of the daughter cells. Such polymer capsules open possibilities for studying cell-cell interactions, fate, and migration patterns while preserving high cell viability and providing intensive fluorescence signals.

Comment 3:
In page 63 the in Figure 2.21 it is nearly impossible to spot the difference between Cy5.5 and Cy3.5 dyes – please highlight the difference if the figure between the two dies. In the same page, the Cy7 is defined as "far-red channel" dye (835 nm). This should read "infrared channel" as 780nm-1mm is defined as infrared range (with 780-2500 nm typically referred as near-infrared range (NIR)).
Change 3:
The difference between Cy5.5 and Cy3.5 dyes was highlighted with ovals in Figure 2.21.
For Cy7, the far-red channel was changed to the infrared channel.

Comment 4:
Concluding remarks to the section on Cyanine dyes are very good (and welcome) in page 64 as they summarise the current understanding of the photoconversion mechanism. However, the following section on Xanthene dyes does not have the corresponding remarks that summarise understanding of photoconversion in that system, which begs the question of why they are even considered.
Change 5:
Concluding remarks have been added to the section 2.3.2.2 (Xanthene dyes) for each subsection and the whole section:
Section "Triphenylmethane derivatives": Therefore, triphenylmethane derivatives experience photoconversion due to the removal of ethyl groups. This property makes them attractive candidates for the production of photoconvertible cell labels.
Section "Diphenylmethane derivatives": Therefore, pyronin dyes and their derivatives potentially exhibit photoconversion properties and might be used for studies on the synthesis of photoconvertible labels.
Section "Hydroxyl derivatives" and whole group: Therefore, fluorescein also has the potential to be applied to the production of photoconvertible labels. To conclude for the whole group of xanthene dyes, there are several promising candidates for photoconvertible cell labels. Based on this literature, rhodamine, pyronin, and fluorescein dyes will be studied for such applications in the thesis.

Comment 6:
In general, I would suggest reviewing the structure of each section of the dissertation to make sure that each chapter, section, subsection etc. contain introductory remarks, concluding remarks summarising the main findings of the chapter/section/subsubsection and a sentence introducing the next part (e.g. "in the next section we will consider THESE, because of THIS and THIS" to maintain the logical flow across the thesis. For instance Section 2.3 ends abruptly in description of mechanism of photoconversion of various dyes, but there are no concluding remarks: what did we learn about the nature of various dye photoconversion and how is this relevant to the goals of this project?
Change 6:
The logical flow of the dissertation has been improved by adding the concluding and introductory remarks in the following Sections and Subsections:

<u>Section 2.1 Polymer microcapsules synthesis</u>

In conclusion, polymer microcapsules are a unique system. The capsule size can be precisely controlled and the choice of polymers for the capsule shell is wide. Capsules have low cytotoxicity and are easily taken up by various cell lines. Capsules can also be loaded with drugs, dyes, and nanoparticles (NPs). These properties make microcapsules attractive for cell-labeling and tracking applications.

Subsection 2.1.2 Multilayer assembly of polymers on spherical templates for capsule formation:
To conclude the part of literature review on the synthesis of polymer microcapsules, this is a complicated yet beautifully simple process. Charge overcompensation of polymers results in the growth of polyelectrolyte layers, creating thin and soft shells for the delivery of various materials. The methods of cargo loading into capsules are discussed in the next section.

Subsubsection 2.1.3.1 Pre-loading:
Therefore, the choice of pre-loading technique depends on the type of cargo, solvent, and need for low or high encapsulation values.

Subsubsection 2.1.3.1 Post-loading:
Such a variety of loading methods provides great opportunities for material encapsulation depending on the researcher's goal, capsule composition, and actual cargo.

Subsection 2.1.4 Properties of microcapsules
In this section, the properties of microcapsules are discussed from a cellular point of view. It is crucial to understand the mechanism of capsule internalization and the various impacts of capsules on cells to provide effective labeling and tracking.
In conclusion, polymer microcapsules are perfect candidates for various cell applications. Capsules have low cytotoxicity and a soft shell composition, which compliments their effective uptake. Mentioned features open possibilities for the delivery of various cargoes to cells, such as nanoparticles, dyes, and drugs.

Subsection 2.1.5 Biomedical applications of polymer microcapsules
In the next section, various biological applications of microcapsules are discussed to create a vision for capsule application possibilities.

Section 2.2 Carbon nanodots
In this section, carbon nanodots (CNDs) are discussed because they played a role in the photoconversion of polymer capsules, reported previously [24], which inspired the current work.
In conclusion, CNDs are non-toxic materials that can be easily produced from various sugar sources. Their unique optical properties make them attractive candidates for photocatalytic applications. In addition, their nontoxicity is favorable for biological applications.

Subsection 2.2.1 Carbon nanodots production
Therefore, bottom-up synthesis is more suitable for the production of CNDs precisely in the capsule shell, which was done previously [24]. In the next section, the properties of CNDs will be discussed.

Subsection 2.2.2 Carbon nanodots properties
In conclusion, CNDs have unique optical properties, which highly depend on the size, surface passivation, and environment's pH. Therefore, their synthesis parameters should be carefully controlled to ensure the reproducibility of the results. In the next section, the applications of CNDs will be discussed.

Subsection 2.2.3 Carbon nanodots applications
In conclusion, CNDs are non-toxic materials that can be easily produced from various sugar sources. Their unique optical properties make them attractive candidates for photocatalytic applications. In addition, their nontoxicity is favorable for biological applications. In the next section, the photoconversion phenomenon will be discussed.

Section 2.3 Photoconversion
In conclusion, the photoconversion mechanism of various FPs and dyes was discussed. While FPs are widely used for cell labeling, various organic fluorescent dyes also exhibit potential for the production of

capsule-based photoconvertible labels, such as cyanine, rhodamine, acridine, pyronine, which will be used for further studies. In the next section, cell labeling and tracking techniques are discussed.

Subsection 2.3.1 Photoconversion mechanism in fluorescent proteins
In conclusion, photoconvertible FPs have a unique chemical structure that consists of proteins and chromophores, making photoconversion possible in such system. Oxygen also plays a crucial role in the photoconversion reaction.

Subsection 2.3.2 Photoconversion mechanism in organic dyes
Several organic fluorophores can also be photoconverted and used for cell labeling. Unlike FPs, organic fluorophores do not require genetic modification of the cells [178]. In this section, the photoconversions of cyanine, xanthene, and acridine dyes are discussed.

Comment 7:
I also found it odd that subsubsections (describing specific dyes) of subsection 2.3.2 are not enumerated, hence the corresponding parts do not appear in the Contents.
Change 7:
Subsubsections are now enumerated and appear in the Contents.

Comment 8:
In page 77 there is generic statement "Nevertheless, such fluorescent markers have their disadvantages and limitations that should be taken into account, such as leakage, dilution with cell division, photobleaching, and cytotoxicity [21,22]. Also, staining of the nearby non-labeled cells is common [23], potentially distorting research results." This is far too generic and it is not clear why one should worry about any of these and go onto development of alternatives. In other words, how these existing problems prevent further progress in relevant fields and what are the practical consequences of distorting the results (e.g. to patient health, hypothesis testing, development of new theories and/or methodologies etc.)?
Change 8:
The following text has been added in Section 2.4.2 (Organic fluorophores):
Overall, the discussed challenges restrict the applicability of traditional optical labeling with non-covalently binding dyes in free form due to possible distortions in the interpretation of research results. Thus, leakage and dilution of the dye impact the accuracy and efficiency of long-term tracking studies, potentially leading to unreliable data and misinterpretations of cell behaviour over time. Addressing these issues is vital for improving research outcomes, enhancing our understanding of cellular mechanisms, and ensuring the safe and effective application of cell-based therapies in clinical settings.

Comment 9:
In page 103 in third line the reference to the figure is missing – it appears as "Figure ??"
Change 9:
The reference to the Figure has been fixed.

Comment 10:
In page 111 the units are missing in line 3 "± 0.5" – presumably microns? In the same line "which is almost two times less than that the initial capsules." should be changed to "which is almost half the size of the initial capsules."
Change 10:
Microns have been added to 3 ± 0.5 μm. The phrase has been changed to "which is almost half the size of the initial capsules."

Comment 11:
In page 124 one of the dyes is abbreviated as PyB in the text, while it appears as PyrB in Figure 4.16.
Change 11:
The abbreviation of PyrB was corrected to PyB in Figure 4.16.

Comment 12:
In page 128 it is reported that "The increased signal after photoconversion appeared in the range of 505–540 nm for RhB and Rh6G excited by the laser with a 488 nm wavelength. At the same time, no such

effect was observed for PyB." It would be good to have some lines in the text to explain/hypothesise why no such effect was observed in PyB as it seems to contradict to the data in Fig 4.20j where there is an effect of increased intensity in 505-540 window.
Change 12:
The explanation of this phenomenon was added to Section 4.7:
Supposedly, a blue shift is present owing to the decrease in the signal in the red channel. In other words, because part of the spectral signal was detected in the green channel (505–540 nm) before photoconversion, it became more prominent after the bleaching of the dye in the red channel. This effect resulted in a blue shift of capsule spectra.

Comment 13:
In Fig 4.21 it would be useful to label the graphs with the names of the corresponding dyes for clarity.
Change 13:
In Figure 4.21 Graphs have been labeled with the names of the corresponding dyes.

Comment 14:
In page 132 change "Also, after the following UV irradiation…" to "Also, after the UV irradiation…".
Change 14:
The phrase has been changed to "Also, after the UV irradiation…".

Comment 15:
In the concluding section (Chapter 7) it is stated that "The encapsulated dyes exhibited robust fluorescence after thermal treatment, except for TR, which did not withstand high-temperature treatment." I think it is essential to add information on the temperature range as otherwise the reader has to search for the answers throughout the thesis.
Change 15:
Temperature values were added in Chapter 7 in section General conclusion:
The encapsulated dyes exhibited robust fluorescence after thermal treatment, except for TR, which did not withstand high-temperature treatment for the dye encapsulation (180 ◦C).

Comment 16:
In the same page (183) the statement that "RhB and Rh6G were found to be photostable and had remarkable photoconversion properties …" is far too generic: in what way are the found properties are remarkable – e.g. perhaps it would be important to mention at this point that photoconversion can be done with some colour/wavelength precision, enabling individual labelling of cells in large populations and/or multi-coding when more than one capsule is internalised by a cell, etc.
Change 16:
The word "remarkable" was removed to increase the clarity of the text. The potential applications of the capsules are listed in the same section (General conclusions).

Comment 17:
In the same page there is another very generic statement that "…detection parameters are more suitable for the Leica confocal microscope laser set. ". In what way are they "more suitable"? Please provide some details. Actually, I don't think this is do with Leica system per in general, but perhaps the specific Leica system used in this work.
Change 17:
The statement "…detection parameters are more suitable for the Leica confocal microscope laser set." were removed to avoid ambiguity. Originally, it was about the specific lasers of our Leica microscope, but detection parameters are expected to be suitable for the lasers of other confocal microscopes.

Professor Alexander Korsunsky

Comment 1
Why were thermally treated microcapsules chosen for cell experiments?
What are the advantages and disadvantages of both types of capsules prepared with thermal and non-thermal approaches?

Change 1:
Both types of capsules prepared with thermal (Chapter 4) and non-thermal treatment (Chapter 5) are expected to be applicable for cell tracking. In this chapter, capsules prepared with thermal treatment were chosen for cell experiments for several reasons. The decrease in size during synthesis leads to the increase in shell thickness and stiffness. Therefore, capsules can be observed as separate bright “beads” in the cells that do not deform and do not collapse with each other. A smaller diameter of capsules is also beneficial for uptake by smaller cells (for example, fibroblasts [299]). At the same time, regular LbL capsules prepared without thermal treatment can deform due to their thin soft shell [300]. This might lead to their adhesion to each other and complicate the distinction of separate capsules in the cell for effective labeling. Note that such LbL capsules potentially could be used for drug delivery since no heating is required for their production, which is crucial for the drug. Moreover, this synthesis technique is suitable for the dyes that do not withstand thermal treatment. Nevertheless, for the goals of this research, thermally treated capsules were chosen for labeling experiments, taking into account their discussed advantages over LbL capsules.
This text has been added in Chapter 6 (Introduction).

Comment 2:
In the literature review, the candidate may elaborate further on the disadvantages of fluorescent proteins and dyes. Why is there a need for an alternative labelling approach?
Change 2:
The following text has been added in Section 2.4.1 Fluorescent proteins:
To conclude, photoconvertible FPs are a powerful tool for marking cells and observing changes in the selected cells over time. This approach offers the possibility of marking and re-sorting cells with certain requested characteristics for cultivation and growth (e.g., creation of purified cell lines) [216]. Nevertheless, this approach has its limitations: (i) some FPs exhibit toxicity [18] and a tendency to aggregate [16], (ii) a gradual decrease in the photoconverted fluorescent signal during cell doubling (i.e., daughter cells share a part of the converted protein among themselves until the signal disappears completely) [217], (iii) the inability to identify an individual cell among the marked ones, and (iv) the need for gene modification of the primary culture carrying the gene of the photoconvertible protein [164]. Gene modification is the main limitation to exploiting the resulting cell clones in personalized medicine because of the probability of random mutations occurring during transfection.
The following text has been added in Section 2.4.2 Organic fluorophores:
Overall, such a variety of fluorescent photoconvertible dyes provides a wide range of fluorescence detection options. The discussed dyes often have bright fluorescence signals, and the staining procedure is quite simple [219]. Nevertheless, these dyes possess certain limitations. For example, DiI is highly cytotoxic, and its safe concentration greatly depends on the cell type [220]. Without a predefined safe concentration of the dye, dead cells might appear during culture preparation. Such debris from the dead cells would be taken up by other non-labeled cells, staining them and potentially distorting research results. In cases where there are no dead cells, DiI can still diffuse from labeled cells and stain other cell lines [221]. The same tendency was reported for Hoechst dye [222]. When labeled and nonlabeled cells were co-cultivated, the transfer of this dye to more than 50% of non-labeled cells was observed within six hours after the start of the experiment. DAPI also stains non-labeled cells due to non-covalent binding to DNA [223]. Moreover, it does not penetrate the cell membrane effectively and cannot be used at higher concentrations due to cytotoxicity, which limits its labeling possibilities [224]. Additionally, dyes are diluted due to cell division, which leads to a decrease in the fluorescence intensity signal. Therefore, tracking is possible for up to three divisions [208].
Cyanine dyes can also exhibit cytotoxic properties [225], and the possibility of their migration into neighboring unstained cells during co-cultivation has not been sufficiently studied. At the same time, their advantages include the potential for covalent cross-linking with biological molecules [226], as well as a wide choice of dyes with different excitation and fluorescence ranges, including those for nearinfrared (NIR) (Cy 5.5, Cy7). Therefore, the development of a new form of the dye for cell labeling is required, which will be demonstrated in Chapter 5.
To conclude, the discussed challenges restrict the applicability of traditional optical labeling with non-covalently binding dyes in free form due to possible distortions in the interpretation of research results. Thus, leakage and dilution of the dye impact the accuracy and efficiency of long-term tracking studies, potentially leading to unreliable data and misinterpretations of cell behaviour over time.

Addressing these issues is vital for improving research outcomes, enhancing our understanding of cellular mechanisms, and ensuring the safe and effective application of cell-based therapies in clinical settings.

Professor Alexey Yashchenok

Comment 1:
Page 5 – It would be more informative to add whether the conference presentation was oral or poster.
Change 1:
In the Conferences section, information about the format of the presentation was added for each conference (4 oral reports and 2 posters)

Comment 2:
Page 13 - The abbreviation “MS- mass-spectrometry” is mentioned twice.
Change 2:
One MS-mass spectrometry has been deleted.

Comment 3:
Page 27 - “3. Analyze cytotoxicity and uptake of capsules, prepared with a a hydrothermal approach.”. Please correct.
Change 3:
The phrase was corrected: “Analyze cytotoxicity and uptake of capsules prepared with the hydrothermal approach”.

Comment 4:
Page 47 - “In could be achieved by…”. Please correct.
Change 4:
The phrase was corrected: “This can be achieved…”

Comment 5:
Page 50 - “The absortion peak around 220-270 nm…”. Please correct.
Change 5:
The phrase was corrected: “The absorption peak around 220-270 nm…”

Comment 6:
Page 51 - “As for luminescence emission, the photoluminescence (PL) of CNDs depends on the excitation wavelength (Figure 2.12b) [116].” There is no Figure 2.12b, please check.
Change 6:
Figure 2.12b was corrected to Figure 2.12.

Additional corrections from Zhanna Kozyreva

In the Publications section one more article was added as published:
Polina A. Demina, Olga I. Gusliakova, Zhanna V. Kozyreva, Daria A. Terentyeva, Dmitry A. Gorin, Irina Yu. Goryacheva, Gleb B. Sukhorukov, and Olga A. Sindeeva. Photoconvertible markers for study individual myoblast migration into the macrophage’s colony. Optical Materials, 157:116148, September 2024.
One more article was added as under review:
Zhanna V. Kozyreva, Polina A. Demina, Olga I. Gusliakova, Gleb B. Sukhorukov, and Olga A. Sindeeva. Transferring of cyanine dyes in free and encapsulated form between cells. Journal of Materials Chemistry B (under review)

Conference was added:
6. Zhanna V. Kozyreva, Olga A. Sindeeva, Polina A. Demina, Daria A. Terentyeva, Olga I. Gusliakova, and Gleb B. Sukhorukov. Fluorescent convertible microcapsules as a tool for studying the migration of mouse mesenchymal stromal cells into glioblastoma. XXXVI Symposium "Modern Chemical Physics", Tuapse, 16-26 September 2024. Oral report.

Figure 4.22 was combined from previous Figures 4.22 and 4.23 to optimize the layout of the text and figures.

In materials section, LPD for the excitation and detection of the samples was corrected in green the channel in several subsections:

1. Section “Capsules imaging”: The laser power densities (LPD) for the visualization of the samples were 9 kW/cm2 for Fl, AY, AO, and TR;
2. Section “Photoconversion”: The LPD for visualization in this channel was 9 kW/cm2.
3. Section “Capsules photostability measurements”: …the following LPDs: 9 kW/cm2 for Fl, AY, AO, and TR;