

論文 / 著書情報
Article / Book Information

題目(和文)	
Title(English)	Research on lanthanide doped upconversion nanoparticles for photodynamic therapy
著者(和文)	リウユ
Author(English)	Yu Liu
出典(和文)	学位:博士(理学), 学位授与機関:東京工業大学, 報告番号:甲第9256号, 授与年月日:2013年9月25日, 学位の種別:課程博士, 審査員:湯浅 英哉,関根 光雄,清尾 康志,大窪 章寛,林 宣宏,小倉 俊一郎
Citation(English)	Degree:Doctor (Science), Conferring organization: Tokyo Institute of Technology, Report number:甲第9256号, Conferred date:2013/9/25, Degree Type:Course doctor, Examiner:,,,,,
学位種別(和文)	博士論文
Type(English)	Doctoral Thesis

**Research on lanthanide doped upconversion nanoparticles
for photodynamic therapy**

Mentor: Professor Hideya Yuasa

Life Science

Graduate School of Bioscience and Biotechnology

09D52042

Liu Yu

2013

Tokyo Institute of Technology

Contents

Chapter I	Introduction	1
I-1	Upconversion process	1
I-2	Upconversion nanoparticles	2
I-3	Photodynamic therapy	5
Chapter II	Synthesis of variouslanthanide doped upconversion nanoparticles	19
II-1	Introduction	19
II-2	Gd doped oleic acid coatedupconversion nanoparticles	19
II-3	Boric acid scaffold hexagonal upconversion nanoparticles	26
II-4	EDTA scaffold hexagonal upconversion nanoparticles	30
II-5	Other metal doped upconversion nanoparticles	32
II-6	Summary	33
Chapter III	Sugar-attached upconversion lanthanide nanoparticles	36
III-1	Introduction	36
III-2	Attachment of sugar to upconversion nanoparticles	38
III-2-1	Attachment of sugar to PEI coated UCLNPs	38
III-2-2	Attachment of sugar to OA coated UCLNPs	43
III-3	Lectin assay of sugar-attached UCLNPs	48
III-4	Macrophage assay of sugar-attached UCLNPs	55
III-5	Summary	59
Chapter IV	Using synthesized UCLNP in PDT experiments	63
IV-1	Introduction	63
IV-2	MTT assay of synthesized UCLNPs	65
IV-3	PDT experiments of synthesized UCLNPs	66
IV-4	Summary	67
Chapter V	Conclusion	68
	Experimental section	69
	Acknowledgement	79

Chapter I Introduction

I-1 Upconversion process

The upconversion phenomena were first recognized by Francois Auzel and *et. al.* in the mid-1960s.^[1] The upconversion here refers to an abnormal optical process where successive absorptions of two or more incident photons via intermediate excited states are followed by emission from the higher energy states (shorter wavelengths) than the absorbed light energy (a longer wavelength). Since then, the conversion of near-infrared radiation into a visible radiation has been increasingly attracting the attention of researchers. However, the development of this field at first was mainly on infrared quantum counter detectors, temperature sensors, or compact solid state lasers, being rarely on biological science because upconversion crystals have suffered from their large sizes and poor dispersibility and upconversion emission in water. On the other hand, 21st century has been witnessing tremendous developments in nanotechnology, which have led to the application of upconversion nanoparticles in bioimaging, biolabeling and bioassays.^[2-4]

Upconversion process can be roughly divided into three categories: excited state absorption (ESA), energy transfer upconversion (ETU), and photon avalanche (PA). In all these three processes, two or more photons are absorbed sequentially not simultaneously (Figure I-1).

As for ESA (Figure I-1a), the single ion is excited by two sequential photons. The ion is firstly excited to the metastable level E1 from the ground level G by absorption of one photon, which is known as ground state absorption (GSA). And then, a second pump photon promotes the ion from metastable level E1 to a higher excitation level E2 and result in upconversion emission corresponding to the transition from E2 to G.

Different from ESA that contains only one ion, ETU (Figure I-1b) is caused by two neighboring ions. Each of the two neighboring ions is firstly excited to the metastable level E1 from ground level G. And then a non-radiative energy transfer process excites one of the two ions from metastable level E1 to a higher excitation level E2 letting the other one relax from E1 to ground state G. The ion at E2 relaxes to ground state G resulting in upconversion emission. The average

distance between the two neighboring ions has a strong influence on the upconversion efficiency of the ETU process.

The third category, PA (Figure I-1c) also contains two neighboring ions. One of the two ions is excited to metastable level E1 by non-resonant weak GSA, and then to a higher state E2 in the same way as ESA. Cross-relaxation energy transfer (or ion pair relaxation) occurs between the ion at E2 and a neighboring ion at ground state G, leading both ions to the intermediate metastable level E1. Further cross-relaxation from one to another ion enables generation of E2 by ESA resulting in upconversion emission through the relaxation from E2 to G. Among all three categories, ETU provides the most effective upconversion emission.

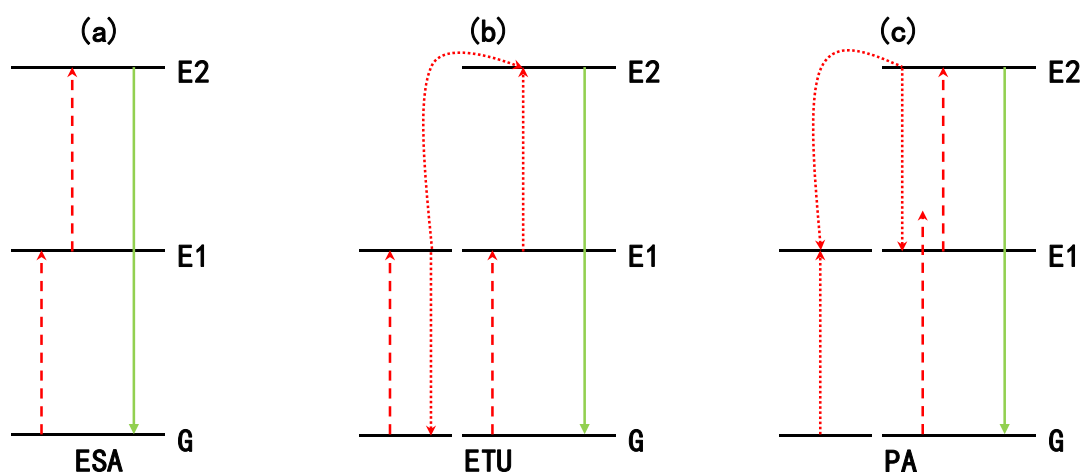


Figure I-1. Three main categories of upconversion processes: (a) ESA, (b) ETU, and (c) PA.

The dashed, dotted, and full arrows represent photon excitation, energy transfer, and emission, respectively as described by F. Wang *et al.* (2009)^[2]

I-2 Upconversion nanoparticles

Most of the inorganic materials can't exhibit upconversion luminescent property at room temperature. Thus in the upconversion nanoparticles, they always contain a crystalline host material with two or more lanthanide dopants added to the host lattice as sensitizer or emitter.

The host materials require lattice matches with dopant ions and low phonon energies. Almost all trivalent rare earth ions have similar ionic size and chemical properties, which are quite suitable to

be used as host materials in upconversion nanoparticles. Also some other ions, such as some alkaline earth ions and some transition metal ions, which have close ionic size to lanthanide ions, can be used as host materials in upconversion nanoparticles. Host materials with low phonon energies can help decrease non-radiative loss and increase the radiative emission. Yttrium is the most common host material in upconversion nanoparticles.

Trivalent ytterbium exhibits extremely favorable property to be used as sensitizer in upconversion nanoparticles because it has quite simple energy level scheme with only one excited 4f level $^2F_{5/2}$ (Figure I-2). The absorption band of Yb^{3+} is around 980 nm and the transition of $^2F_{7/2}$ to $^2F_{5/2}$ is well resonant with many f-f transitions of typical upconverting ions, such as Er^{3+} or Tm^{3+} .

In a typical upconversion process of Yb/Er-based lanthanide nanoparticles (LNPs), Yb^{3+} act as a sensitizer to absorb 980 nm near-infrared (NIR) light transferring energy to the emitter Er^{3+} (Figure I-2). The excited Er^{3+} has a long lifetime because the 4f-4f transitions are forbidden by the Laporte rule and shielded by the filled 5s and 5p orbitals, permitting the second and third excitation to upper energy levels. The emission from these upper energy levels accompanied by non-radiative transitions of small energy gaps gives structured sharp peaks at green and red color regions of the emission spectrum (Figure I-2).

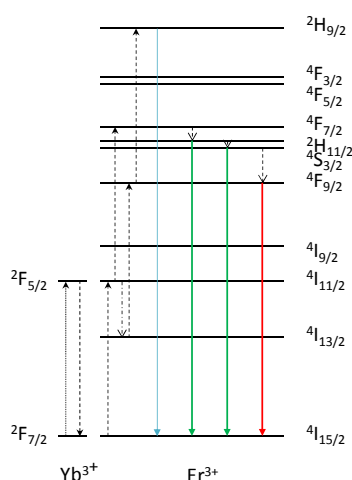


Figure I-2. Proposed energy transfer mechanism showing the upconversion process in Er^{3+} doped crystals under 980 nm diode laser excitation. The dotted, dashed, dashed-dotted, and full arrows represent photon excitation, energy transfer, multiphonon relaxation, and emission processes, respectively as described by F. Wang *et al.*(2009)^[2]

Besides sensitizer and emitter, some other lanthanide dopants can also have influence on synthetic process of upconversion nanoparticles and upconverting luminescence. These dopants can stabilize a specific crystal phase^[5] and emission^[6] properties of nanoparticles. One of such dopants is Gd^{3+} .^[7,8] In $NaREF_4$ (RE represents rare earth ions) crystals, phase transition from cubic to hexagonal occurs with the increase of rare earth ionic radius or polarizability (Figure I-3). In $NaYF_4:Yb/Er/Gd$ upconversion nanoparticles, the size of Gd^{3+} ($r = 1.193 \text{ \AA}$)^[9] is similar but larger than the host Y^{3+} ions ($r = 1.159 \text{ \AA}$).^[9] The dopant Gd^{3+} is able to shorten the synthetic reaction time from several days to two hours, lower the reaction temperature from over 300°C to 200°C , and aid the formation of a pure hexagonal-phase that enables a higher emission intensity.^[7,8]

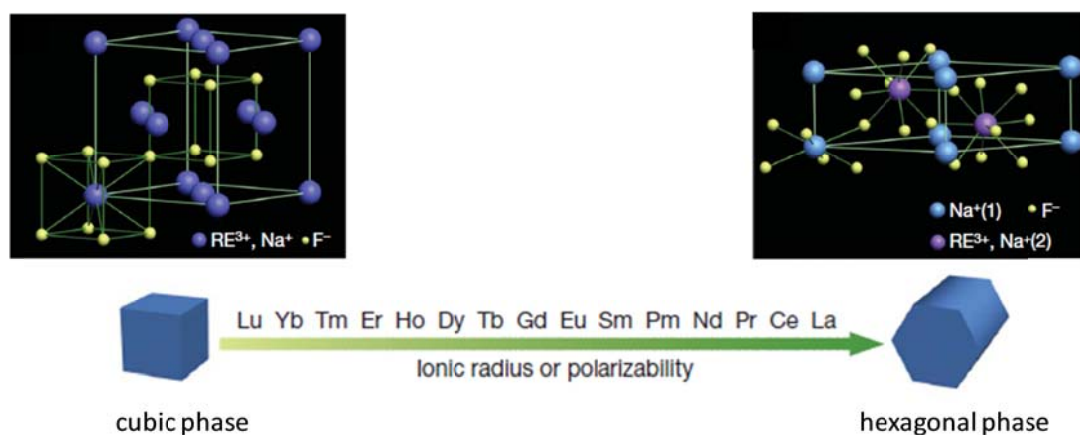


Figure I-3. Phase transition from cubic to hexagonal of $NaREF_4$ crystals as described by F. Wang *et al.* (2010)^[7]

Comparing to organic fluorophores^[10] and semi-conducting nanocrystals (as quantum dots),^[11] the unique upconversion nanoparticles have many advantages, e.g.; the great distinguish ability of signals due to the large anti-Stokes shift and sharp peaks, the absence of autofluorescence from biological samples,^[12] and high penetrativity^[13-17] of NIR excitation light.^[18] LNPs have the additional merits of non-photobleaching and non-photoblinking properties.^[19]

I-3 Photodynamic therapy

Photodynamic therapy (PDT) is a kind of photo medical method using nontoxic light-sensitive compounds which become toxic to targeted cells when they are exposed selectively to light. Most PDT applications contain three main factors: light-sensitive compounds called photosensitizer, proper light source, and tissue oxygen.

Photosensitizer is the key component in PDT. As shown in Figure I-4, photosensitizer can be excited to excited state from ground state by absorption of proper light. With non-radiative energy transfer from the relaxation of photosensitizer from excited state back to ground state, tissue oxygen is excited to singlet oxygen which is toxic to cells and can kill cell through apoptosis or necrosis. Photosensitizers should meet some characteristics: high absorption at longer wavelength; high singlet oxygen quantum yield; low photobleaching; high chemical stability; easy to eliminate; and low toxicity.

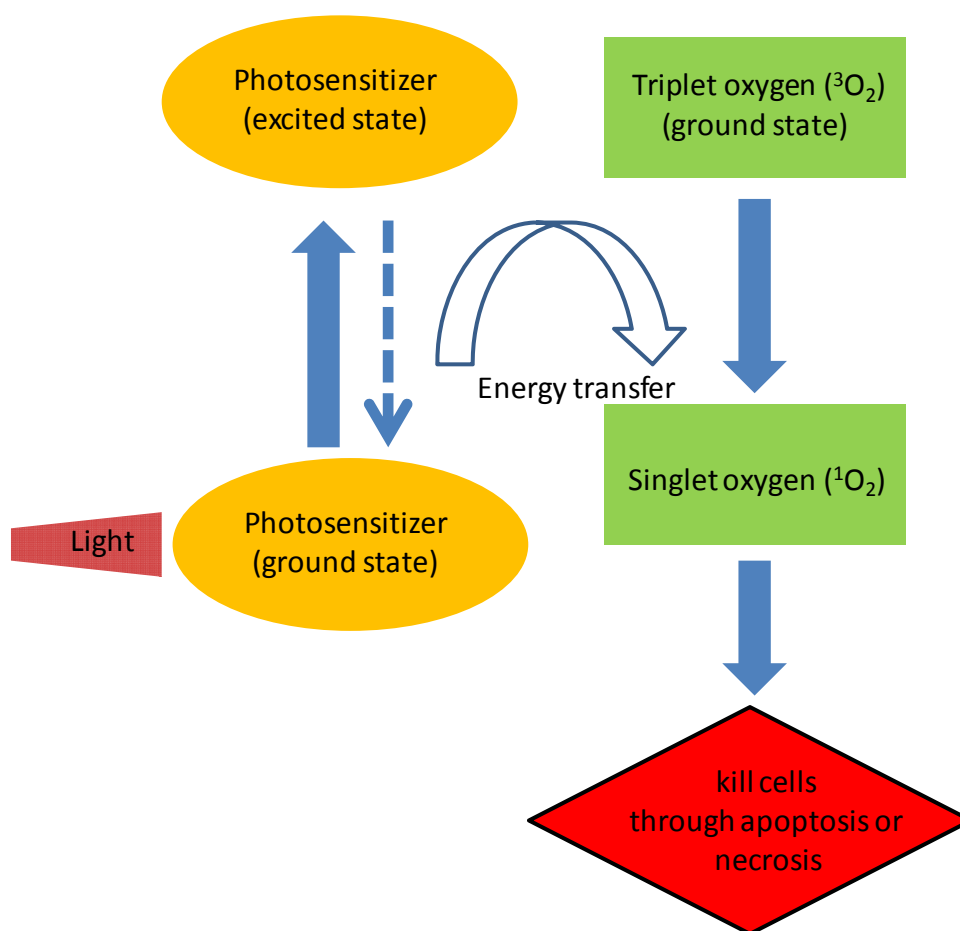


Figure I-4. Basic mechanism of photodynamic therapy.

Photosensitizers can be categorized into three broad families by their chemical structures. They are porphyrin platform, such as hematoporphyrin derivative (HpD), HpD-based, benzoporphyrin derivative (BPD), protoporphyrin IX (PPIX) derived from 5-aminolevulinic acid (ALA), and texaphyrins; chlorophyll platform, such as chlorins, purpurins, and bacteriochlorins; dyes, such as phthalocyanine and naphthalocyanine.^[20]

For example, in our lab, ALA is used as photosensitizer prodrug because ALA is commercially available and can be converted enzymatically to protoporphyrin which can be used as photosensitizer in PDT. In 1990, Kennedy *et al.*^[21] firstly applied topically ALA-based PDT in clinical setting. As shown in Figure I-5, at the very beginning of the heme biosynthetic pathway, ALA is formed from succinyl coenzyme A (CoA) and glycine. At the end is the incorporation of iron into protoporphyrin IX (PPIX), which takes place in the mitochondria under the action of the enzyme ferrochelatase. With the addition of exogenous of ALA, PPIX may accumulate because of the limited capacity of ferrochelatase. Porphobilinogen deaminase is another enzyme of the heme synthesis parthway (catalyzing the formation of uroporphyrinogen form porphobilinogen). Its activity is higher in some tumors,^[22-24] whereas that of ferrochelatase is lower,^[23-29] so that PPIX accumulates with some degree of selectivity in such tumors.^[30] PPIX is just an efficient photosensitizer which can be used in PDT.

PDT involves the systemic or topical application of a photosensitizer. For example, for skin cancer, most published clinical PDT studies used topical ALA-PDT or systemic HpD/Photofrin-PDT from 1990 to 1995.^[30] Although it appears that systemic administration of exogenous ALA at a dose lower than 60 mg/kg(oral) or 30 mg/kg (intravenous) does not result in any neurotoxic symptoms, some patients still suffer from mild, transient nausea or/and transient abnormalities of liver function. Meanwhile topical ALA-PDT can act as efficient as systemic administration of exogenous ALA for skin cancers. In this case topical ALA-PDT was widely used for skin cancer.

The comparison of topical ALA-PDT and systemic HpD/Photofrin-PDT for the treatment of skin cancer was shown in Table 1.^[30]

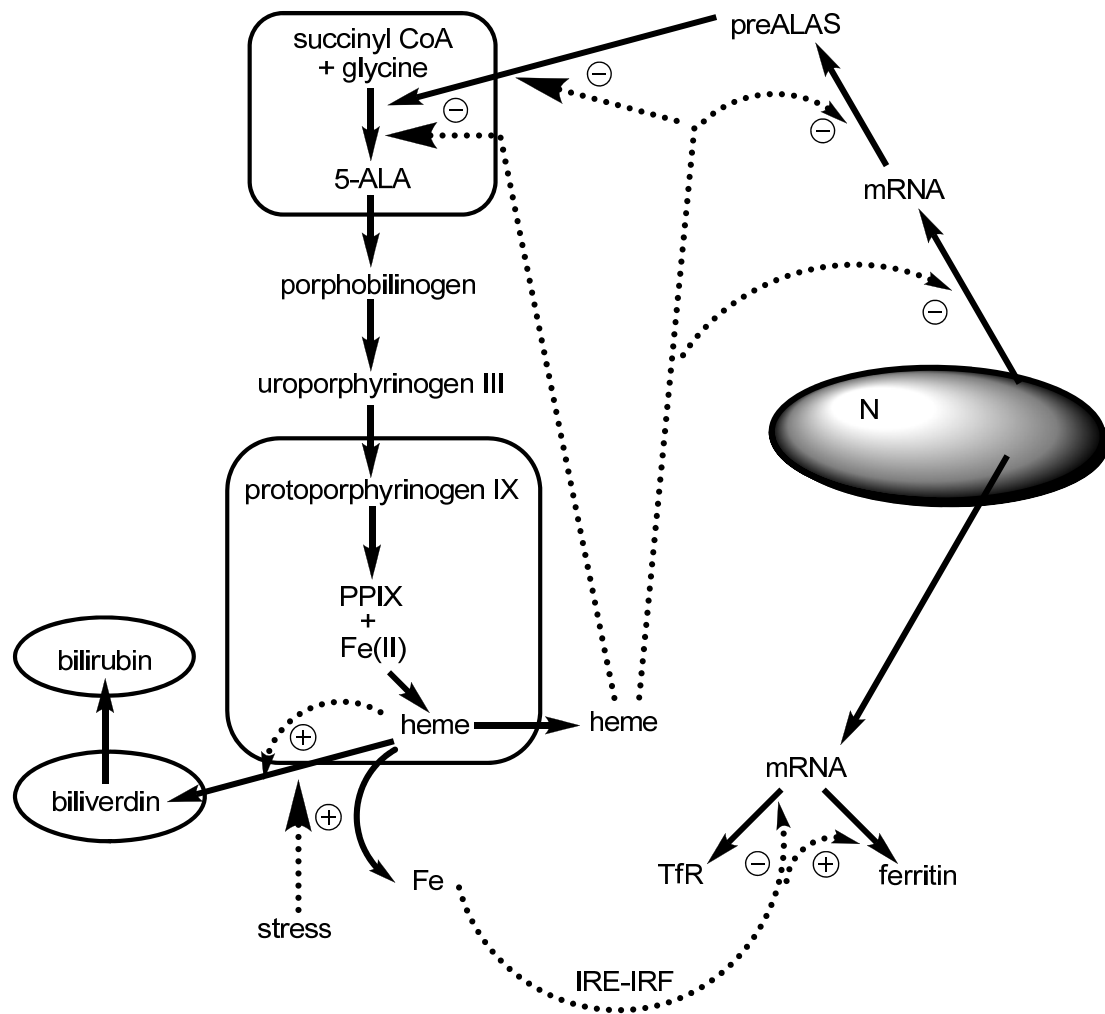


Figure I-5. Regulation of heme synthesis is represented. The synthesis steps are indicated by solid arrows and the regulatory steps by dashed arrows. Encircled “plus” and “minus” signs indicate stimulatory and inhibitory effects, respectively. Mitochondria are indicated as light grey boxes, the nucleus as a dark grey area, and the cytosol as a white area. Bilirubin and biliverdin are located in endoplasmic reticulum. ALAS: ALA synthase; IRE: iron-responsive element; IRF: iron-regulatory factor; TfR: transferrin receptor. This figure is as described by Q. Peng *et al.*(1997)^[30]

Table 1. Comparison of topical ALA-PDT and systemic HpD/Photofrin-PDT for the treatment of skin cancer as described by Q. Peng *et al.* (1997)^[30]

ALA-PDT

- 1) Convenient; available on an outpatient basis
- 2) Low-cost (ALA is cheaper than Photofrin, and ordinary lamps with suitable filters can be used)
- 3) No toxicity or interaction with other medications
- 4) High selectivity leaving the surrounding normal skin intact and functional
- 5) Several separate lesions can be treated simultaneously
- 6) The same lesion(s) can be repeatedly treated
- 7) Cosmetic results are superior to conventional modalities
- 8) No risk of skin photosensitivity after 24 hrs
- 9) Local anesthesia is often required during light exposure
- 10) Efficient for superficial lesions

HpD/Photofrin-PDT

- 1) Relatively inconvenient; patients often stay in the hospital for a few days
 - 2) Expensive (laser is used in most cases)
 - 3) No systemic toxicity or interaction with other medications
 - 4) Selectivity leaving the surrounding normal skin intact and functional
 - 5) Several separate lesions can be treated simultaneously
 - 6) The same lesion(s) can be repeatedly treated
 - 7) Cosmetic results are equal or superior to conventional modalities in most cases
 - 8) Risk of skin photosensitivity for at least 4-6 wks
 - 9) Local anesthesia is sometimes required during light irradiation
 - 10) More efficient than ALA-PDT in treatment of nodular lesions
-
-

Figure I-6 shows the absorption and emission of PPIX. In this figure, the absorption peaks of PPIX are 410, 510, 545, 580, and 635 nm. 410 nm has the highest absorption peak while 635 nm has the lowest. However, most of the ALA-PDT used 635 nm light source with the lowest absorbance^[32-35] which caused low efficiency. The reason of using longer wavelength with lower absorbance instead of shorter wavelength with higher absorbance light source is that the shorter wavelength suffers from low penetration depth. Figure I-7 shows the fluorescence spectra of PPIX at different depths below the human hand surface from 0 to 3 mm under incident light and the penetration depth of light into the human hand as a function of the wavelength.^[30] With the increase of penetration depth, the decrease of fluorescence intensity was quite obvious for shorter wavelength. Unlike shorter wavelength, there was almost no decrease of fluorescence intensity at 635 nm even for 6 mm.

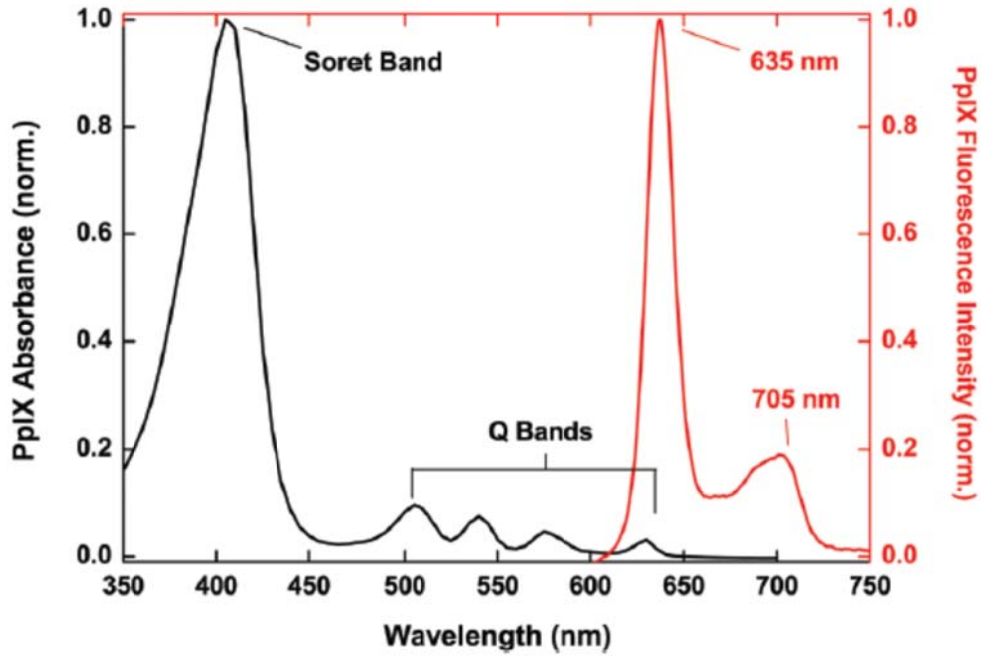


Figure I-6. Broad PPIX absorption spectrum together with characteristic PPIX fluorescence emission with a dominant peak at 635 nm as described by R. M. Valentine *et al.*(2013)^[31]

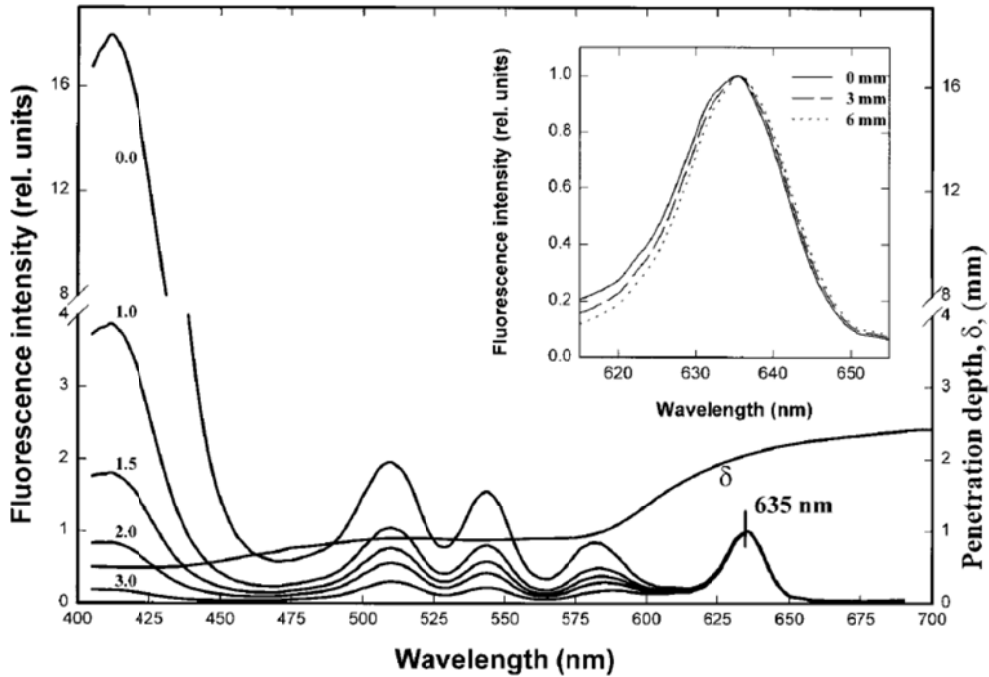


Figure I-7. The fluorescence spectra of PPIX at different depths below the human hand surface from 0 to 3 mm under incident light and the penetration depth (δ) of light into a human hand as a function of the wavelength. The fluorescence intensity was normalized to unity at 635 nm. The inset shows the PPIX fluorescence excitation spectrum in the 620-650 nm wavelength region ($\lambda_{em} = 690$ nm), 0, 3, and 6 mm below the tissue surface. This is described by Q. Peng *et al.*(1997)^[30]

Is there any possibility that PPIX is excited by shorter wavelength light with higher excitation efficiency while still has high penetration depth? The upconversion nanoparticles can convert near-infrared radiation (NIR) into visible light. With this property, upconversion nanoparticles can be applied to photodynamic therapy as shown in Figure I-8. As it is written above, upconversion lanthanide nanoparticles are more proper to be used in PDT than organic fluorophores or semi-conducting nanocrystals (as quantum dots) because LNPs have large anti-Stokes shift and sharp peaks, the ability of absence of autofluorescence from biological samples, high penetration depth, and non-photobleaching and non-photoblinking properties.

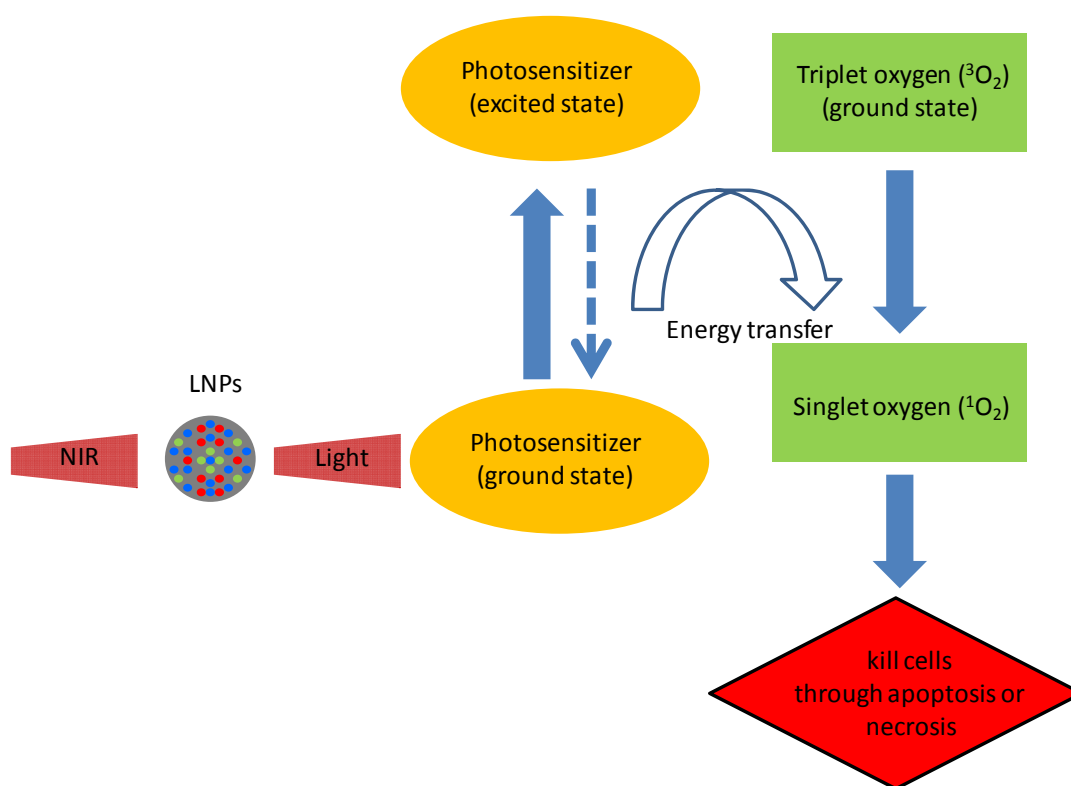


Figure I-8. Upconversion nanoparticles used in PDT.

In summary, we are trying to synthesize the LNPs with the highest and proper emission which can be absorbed by photosensitizer and used in PDT. In order to achieve this goal, the early stage of synthesizing upconversion lanthanide nanoparticles used in photodynamic therapy was studied in this thesis.

This thesis is divided into three chapters.

The first chapter is mainly about synthesizing the upconversion nanoparticles. $\text{NaYF}_4:\text{Yb/Er}$

nanoparticles were used as basic component. Through the investigation of Gd^{3+} , boric acid, Tm^{3+} , and other metal ions as additives, $NaYF_4:Yb/Er/Gd$ nanoparticles were shown to be improved with emission intensity at 540 and 655 nm while boric acid showed obvious increment of emission intensity at also 410 nm. Both of their emission wavelengths were resonated with the absorbance of PPIX and were applicable for PDT. However, boric acid doped LNPs suffered from their large sizes. It was found that further studies of these nanoparticles should be continued.

The second chapter of this thesis is about the synthesis of sugar attached LNPs and the use of them in lectin assay and macrophage assay.

As it is described above, PPIX accumulates with some degree of selectivity in some tumors.^[30] If we want to use the emission of LNPs as light source of PPIX, the concentration of LNPs should also be higher in tumors than in other parts.

Most of tumor-targeted drug delivery systems have been based on the enhanced permeability and retention (EPR) effect of drug-carrying nanoparticles^[36] and/or the binding of antibody-attached drugs to tumor antigens.^[37] An alternative to the expensive and unstable antibodies can be sugar ligands capable of binding to the lectins expressed on the tumor cell surface or tumor-associated macrophage.^[38,39] The sugar-attached LNPs, which are expected to be more stable and cheaper than antibody-attached ones, will ensure the EPR-based tumor targeting delivery of LNPs and endow LNPs with biocompatibility.^[40] For example, mannose is a specific ligand of the mannose receptor^[41,42] of cancer-associated macrophage.^[43,44] By the attachment of mannose on the surface of LNPs, mannose-attached LNPs can be recognized by the mannose receptor on the surface of macrophage so that the concentration of LNPs will be increased in tumors as shown in Figure I-9.

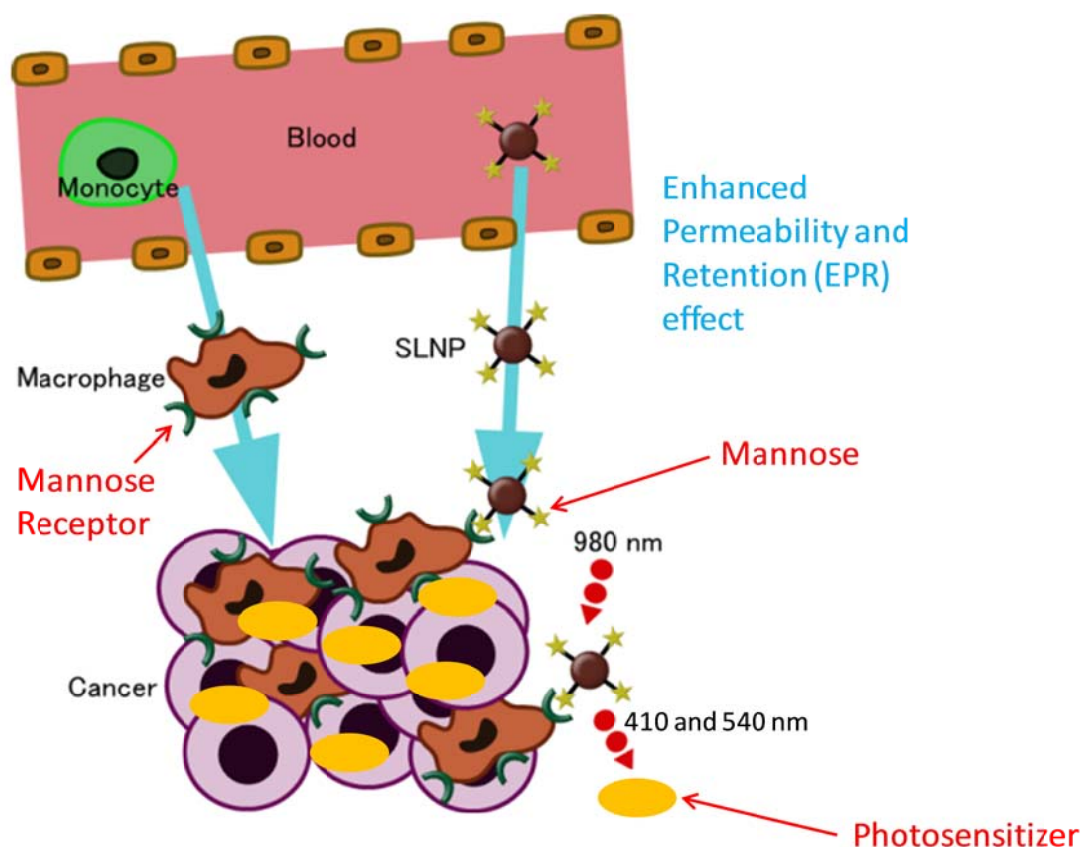


Figure I-9. Sugar attached LNPs aggregated with macrophage in tumors.

In chapter II, mannose and galactose attached LNPs were synthesized. In order to investigate the recognition of sugar-attached LNPs, lectin and macrophage aggregation assays were tested.

The third chapter is about the use of the synthesized LNPs in PDT experiments. The cell toxicity of LNPs was investigated by MTT assay. Finally, the synthesized LNPs were used in PDT experiment in vitro.

References

- [1] F. Auzel, Upconversion and anti-Stokes processes with f and d ions in solids, *Chem. Rev.* **2004**, *104*, 139-173. DOI: 10.1021/cr020357g.
- [2] F. Wang, X. Liu, Recent advances in the chemistry of lanthanide-doped upconversion nanocrystals, *Chem. Soc. Rev.* **2009**, *38*, 976-989. DOI: 10.1039/b809132n.

- [3] F. Wang, D. Banerjee, Y. Liu, X. Chen, X. Liu, Upconversion nanoparticles in biological labeling, imaging, and therapy, *Analyst***2010**, *135*, 1839-1854. DOI: 10.1039/c0an00144a.
- [4] J. Zhou, Z. Liu, F. Li, Upconversion nanophosphors for small-animal imaging, *Chem. Soc. Rev.***2012**, *41*, 1323-1349. DOI: 10.1039/c1cs15187h.
- [5] X. Feng, D. C. Sayle, Z. L. Wang, M. S. Paras, B. Santora, A. C. Sutorik, T. X. T. Sayle, Y. Yang, Y. Ding, X. Wang, Y.-S. Her, Converting ceria polyhedral nanoparticles into single-crystal nanospheres, *Science***2006**, *312*, 1504-1508. DOI: 10.1126/science.1125767.
- [6] Q. Huang, J. Yu, E. Ma, K. Lin, Synthesis and characterization of highly efficient near-Infrared upconversion $\text{Sc}^{3+}/\text{Er}^{3+}/\text{Yb}^{3+}$ tridoped NaYF_4 , *J. Phys. Chem. C***2010**, *114*, 4719-4724. DOI: 10.1021/jp908645h.
- [7] F. Wang, Y. Han, C. S. Lim, Y. Lu, J. Wang, J. Xu, H. Chen, C. Zhang, M. Hong, X. Liu, Simultaneous phase and size control of upconversion nanocrystals through lanthanide doping, *Nature***2010**, *463*, 1061-1065. DOI: 10.1038/nature08777.
- [8] F. Chen, W. Bu, S. Zhang, X. Liu, J. Liu, H. Xing, Q. Xiao, L. Zhou, W. Peng, L. Wang, J. Shi, Positive and negative lattice shielding effects co-existing in Gd (III) ion doped bifunctional upconversion nanoprobes, *Adv. Funct. Mater.***2011**, *21*, 4285-4294. DOI: 10.1002/adfm.201101663.
- [9] R. D. Shannon, Revised effective ionic radii and systematic studies of interatomic distances in halides and chalcogenides, *Acta Crystallogr. A***1976**, *32*, 751-767.
- [10] J. Wang, Q. Chen, N. Bian, F. Yang, J. Sun, A. Qi, C. Yan, B. Han, Sugar-bearing tetraphenylethylene: novel fluorescent probe for studies of carbohydrate-protein interaction based on aggregation-induced emission, *Org. Biomol. Chem.***2011**, *9*, 2219-2226. DOI:

10.1039/C0OB00680G.

[11] J. M. Fuente, S. Penades, Glyco-quantum dots: a new luminescent system with multivalent carbohydrate display, *Tetrahedron: Asymmetry* **2005**, *16*, 387-391. DOI: 10.1016/j.tetasy.2004.12.002.

[12] M. Yu, F. Li, Z. Chen, H. Hu, C. Zhan, H. Yang, C. Huang, Laser scanning up-conversion luminescence microscopy for imaging cells labeled with rare-earth nanophosphors, *Anal. Chem.* **2009**, *81*, 930-935. DOI: 10.1021/ac802072d.

[13] M. Nyk, R. Kumar, T. Y. Ohulchanskyy, E. J. Bergey, P. N. Prasad, High contrast in vitro and in vivo photoluminescence bioimaging using near infrared to near infrared up-conversion in Tm^{3+} and Yb^{3+} doped fluoride nanophosphors, *Nano. Lett.* **2008**, *8*, 3834-3838. DOI: 10.1021/nl802223f.

[14] N. M. Idris, Z. Li, L. Ye, E. K. W. Sim, R. Mahendran, P. C.-L. Ho, Y. Zhang, Tracking transplanted cells in live animal using upconversion fluorescent nanoparticles, *Biomaterials* **2009**, *30*, 5104-5113. DOI: 10.1016/j.biomaterials.2009.05.062.

[15] Q. Zhang, J. Qian, H. Liang, G. Somesfalean, D. Wang, S. He, Z. Zhang, S. Andersson-Engels, Using 915 nm laser excited $\text{Tm}^{3+}/\text{Er}^{3+}/\text{Ho}^{3+}$ -doped NaYbF_4 upconversion nanoparticles for in vitro and deeper in vivo bioimaging without overheating irradiation, *ACS Nano* **2011**, *5*, 3744-3757. DOI: 10.1021/nn200110j.

[16] Q. Liu, Y. Sun, T. Yang, W. Feng, C. Li, F. Li, Sub-10 nm hexagonal lanthanide-doped NaLuF_4 upconversion nanocrystals for sensitive bioimaging in vivo, *J. Am. Chem. Soc.* **2011**, *133*, 17122-17125. DOI: dx.doi.org/10.1021/ja207078s.

[17] J. Wang, F. Wang, C. Wang, Z. Liu, X. Liu, Single-band upconversion emission in lanthanide-doped KMnF_3 nanocrystals, *Angew. Chem. Int. Ed.* **2011**, *50*, 10369-10372. DOI: 10.1002/anie.201104192.

- [18] V. J. Pansare, S. Hejazi, W. J. Faenza, R. K. Prud'homme, Review of long-wavelength optical and NIR imaging materials: contrast agents, fluorophores, and multifunctional nano carriers, *Chem. Mater.***2012**, 24, 812-827. DOI: [dx.doi.org/10.1021/cm2028367](https://doi.org/10.1021/cm2028367).
- [19] S. Wu, G. Han, D. J. Milliron, S. Aloni, V. Altoe, D. V. Talapin, B. E. Cohen, P. J. Schuck, Non-blinking and photostable upconverted luminescence from single lanthanide doped nanocrystals, *Proc. Natl. Acad. Sci.***2009**, 106, 10917-10921. DOI: [10.1073/pnas.0904792106](https://doi.org/10.1073/pnas.0904792106).
- [20] R. R. Allison, G. H. Downie, R. Cuenca, X.-H. Hu, C. JH. Childs, C. H. Sibata, Photosensitizers in clinical PDT, *Photodiagnosis and photodynamic***2004**, 1, 27-42. DOI: [10.1016/S1572-1000\(04\)00007-9](https://doi.org/10.1016/S1572-1000(04)00007-9).
- [21] J. C. Kennedy, R. H. Pottier, D. C. Pross, Photodynamic therapy with endogenous protoporphyrin IX: basic principles and present clinical experience, *J. Photochem. Photobiol. B***1990**, 6, 143–148.
- [22] L. Leibovici, N. Schoenfeld, H. A. Yehoshua, R. Mamet, E. Rakowski, A. Shindel, A. Atsumon, Activity of porphobilinogen deaminase in peripheral blood mononuclear cells of patients with metastatic cancer, *Cancer***1988**, 62, 2297-2300.
- [23] N. Schoenfeld, O. Epstein, M. Lahav, R. Mamet, M. Shaklai, A. Atsmon, The heme biosynthetic pathway in lymphocytes of patients with malignant lymphoproliferative disorders, *Cancer Lett.***1988**, 43, 43-48. DOI: [10.1016/0304-3835\(88\)90211-X](https://doi.org/10.1016/0304-3835(88)90211-X).
- [24] M. Kondo, N. Hirota, T. Takaoka, M. Kajiwara, Heme-biosynthetic enzyme activities and porphyrin accumulation in normal liver and hepatoma cell lines of rat, *Cell Biol. Toxicol.***1993**, 9, 95-105. DOI: [10.1007/BF00755143](https://doi.org/10.1007/BF00755143).

- [25] G. F. Rubino, L. Rasetti, Porphyrin metabolism in human neoplastic tissues, *Panminerva Med.* **1966**, 8, 290-292.
- [26] H. A. Dailey, A. Smith, Differential interaction of porphyrins used in photoradiation therapy with ferrochelatase, *Biochem. J.* **1984**, 223, 441-445.
- [27] A. Smith, Mechanisms of toxicity of photoactivated artificial porphyrins. Role of porphyrin-protein interactions, *Ann. N. Y. Acad. Sci.* **1987**, 514, 309-322.
- [28] M. M. H. El-Sharabasy, A. M. El-Wassel, M. M. Hafez, S. A. Salim, Porphyrin metabolism in some malignant diseases, *Br. J. Cancer* **1992**, 65, 409-412.
- [29] R. Hillegersberg, J. W. Berg, W. J. Kort, O. T. Terpstra, J. H. Wilson, Interstitial photodynamic therapy in a rat liver metastasis model, *Gastroenterology* **1992**, 103, 647-651.
- [30] Q. Peng, T. Warloe, K. Berg, J. Moan, M. Kongshaug, K.-E. Giercksky, J. M. Nesland, 5-Aminolevulinic acid-based photodynamic therapy, 5-Aminolevulinic acid-based photodynamic therapy, *Cancer* **1997**, 79, 2282-2308. DOI: 10.1002/(SICI)1097-0142(19970615)79:12<2282::AID-CNCR2>3.0.CO;2-O
- [31] R. M. Valentine, S. H. Ibbotson, K. Wood, C. T. A. Brown, H. Moseley, Modelling fluorescence in clinical photodynamic therapy, *Photochem. Photobiol. Sci.* **2013**, 12, 203-213. DOI: 10.1039/C2PP25271F.
- [32] F. Cairnduff, M. R. Stringer, E. J. Hudson, D. V. Ash, S. B. Brown, Superficial photodynamic therapy with topical 5-aminolaevulinic acid for superficial primary and secondary skin cancer, *Br. J. Cancer* **1994**, 69, 605-608. DOI: 10.1038/bjc.1994.112.
- [33] K. Svanberg, T. Andersson, D. Killander, I. Wang, U. Stenram, S. Anersson-Engels, R. Berg, J. Johansson, S. Svanberg, Photodynamic therapy of non-melanoma malignant tumours of the skin

using topical delta-amino levulinic acid sensitization and laser irradiation,*Br. J. Dermatol.***1994**, 130, 743-751.

[34] T. Warloe, Q. Peng, H. Heyerdahl, J. Moan, H. B. Steen, K. E. Giercksky, Photodynamic therapy of malignant mesothelioma of pleura, *Proc. SPIE***1995**, 2371, 226-235.
DOI:10.1117/12.203444.

[35] P. G. Calzavara-Pinton, Repetitive photodynamic therapy with topical -aminolaevulinic acid as an appropriate approach to the routine treatment of superficial non-melanoma skin tumours, *J. Photochem. Photobiol. B***1995**, 29, 53-57.

[36] D. Peer, J. M. Karp, S. Hong, O. C. Farokhzad, R. Margalit, R. Langer, Nanocarriers as an emerging platform for cancer therapy, *Nat. Nanotech.***2007**, 2, 751-760. DOI:10.1038/nnano.2007.387

[37] A. M. Scott, J. D. Wolchok, L. J. Old, Antibody therapy of cancer, *Nat. Rev. Cancer***2012**, 12, 278-287. DOI:10.1038/nrc3236.

[38] S. A. Linehan, L. Martinez-Pomares, S. Gordon, Macrophage lectins in host defence, *Microbes Infect.***2000**, 2, 279-288.

[39] K. Jain, P. Kesharwani, U. Gupta, N. K. Jain, A review of glycosylated carriers for drug delivery, *Biomaterials***2012**, 33, 4166-4186. DOI:10.1016/j.biomaterials.2012.02.033.

[40] N. Kamaly, Z. Xiao, P. M. Valencia, A. F. Radovic-Moreno, O. C. Farokhzad, Targeted polymeric therapeutic nanoparticles: design, development and clinical translation, *Chem. Soc. Rev.***2012**, 41, 2971-3010. DOI: 10.1039/c2cs15344k.

[41] L. East, C. M. Isache, The mannose receptor family, *Biochim. Biophys. Acta***2002**, 1572, 364-386.

- [42] P. R. Taylor, S. Gordon, L. Martinez-Pomares, The mannose receptor: linking homeostasis and immunity through sugar recognition, *Trends Immunol.* **2005**, 26, 104-110. DOI:10.1016/j.it.2004.12.001.
- [43] A. Sica, T. Schioppa, A. Mantovani, P. Allavena, Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy, *Eur. J. Cancer* **2006**, 42, 717-727. DOI:10.1016/j.ejca.2006.01.003.
- [44] A. Schmieder, J. Michel, K. Schonhaar, S. Goerdt, K. Schledzewski, Differentiation and gene expression profile of tumor-associated macrophages, *Semin. Cancer Bio.* **2012**, 22, 289-297. DOI:10.1016/j.semcancer.2012.02.002.

Chapter II Synthesis of variouslanthanide doped upconversion nanoparticles

II-1 Introduction

In the previous work done by Mr. Kobayashi in our lab, PEI coated NaYF₄:Yb/Er(10/2 mol%) were synthesized. However, PEI coated NaYF₄:Yb/Er(10/2 mol%) nanoparticles were mostly cubic nanoparticles and showed a relatively weak luminescent intensity. In order to improve the luminescent intensity, composition of upconversion nanoparticles, additives and synthetic protocols should be well defined. In this chapter, the effect of Gd, boric acid, and other metal dopants in the synthesis of upconversion nanoparticles, the synthetic protocol of PEI scaffold upconversion nanoparticles, and the annealing effect of upconversion nanoparticles were investigated.

II-2 Gd doped oleic acid coatedupconversion nanoparticles

In the previous work done by F. Wang et. al.^[1] and F. Chen. et. al.^[2], Gd³⁺ was able to shorten the synthetic reaction time, lower the reaction temperature, and aid the formation of a pure hexagonal-phase that enables a high emission intensity.

In order to investigate the influence of the amount of doped Gd, 30%, 45%, 60% and 75% of Gd³⁺ were doped in NaYF₄:Yb/Er(18/2 mol%) upconversion nanoparticles. In a typical procedure as previously reported by F. Wang et. al.^[1], to synthesize lanthanide-doped NaYF₄ nanoparticles, a DI water solution (3 mL) of 0.6 g NaOH was mixed with 10 mL of ethanol and 10 mL of oleic acid under stirring. To the resulting mixture were selectively added 4 mL of RECl₃ (0.2 M, RE = Y, Yb, Er and Gd) and 2 mL of NH₄F (2 M). Then the solution was transferred into autoclave and heated at 200 °C for 2 h. The obtained nanoparticles were collected by centrifugation, washed with water and ethanol several times, and finally dried under vacuo.

The XRD pattern of the NaYF₄:Yb/Er/Gd(18/2/30 mol%) upconversion nanoparticles is presented in Figure II-1. All of the diffraction peaks were readily assigned to those of hexagonal NaYF₄:Yb/Er (JCPDS 28-1192).

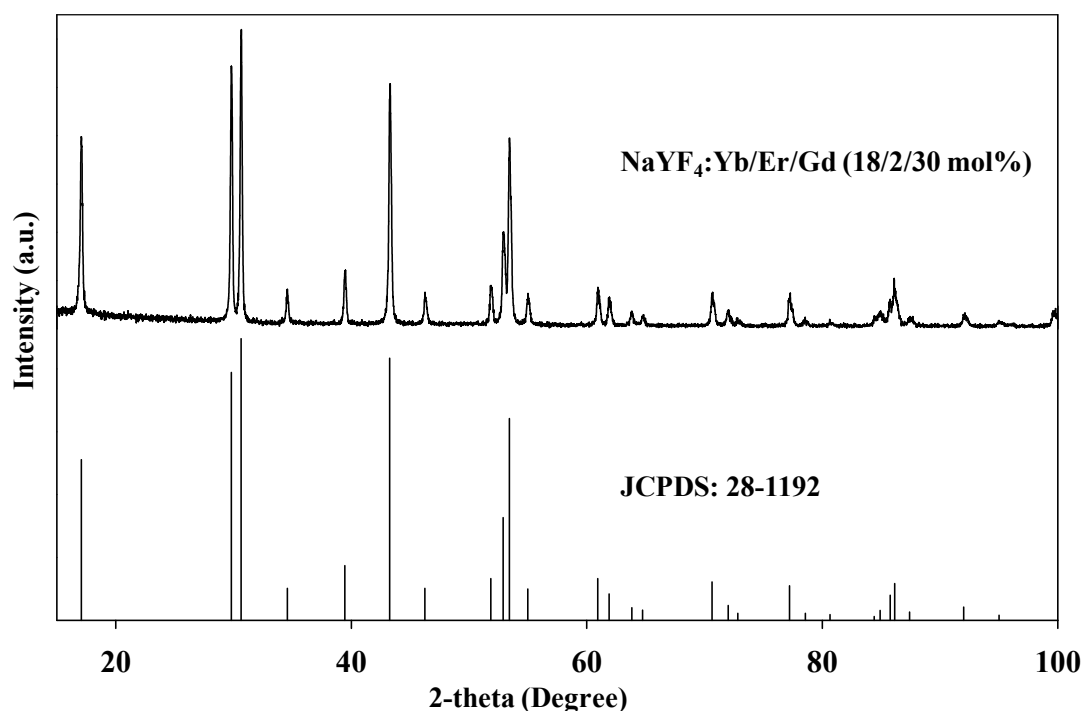


Figure II-1. XRD pattern of nanosized NaYF₄:Yb/Er/Gd (18/2/30 mol%)

The SEM images (Figure II-2) of 30%, 45%, 60%, and 75% doped Gd NaYF₄:Yb/Er(18/2 mol%) nanoparticles indicated that all nanoparticles were homogeneous nanorods. 30% Gd doped nanorods measured around 300 nm in length and 50 nm in width. 45% Gd doped nanorods measured around 200 nm in length and 25 nm in width. And 60% Gd doped and 75% Gd doped ones measured around 100 nm and 50 nm in lengths and 15 nm and 10 nm in widths, respectively. With the increase of Gd doped amount, the size of nanorods was decreased and the morphology of nanoparticles was not changed.

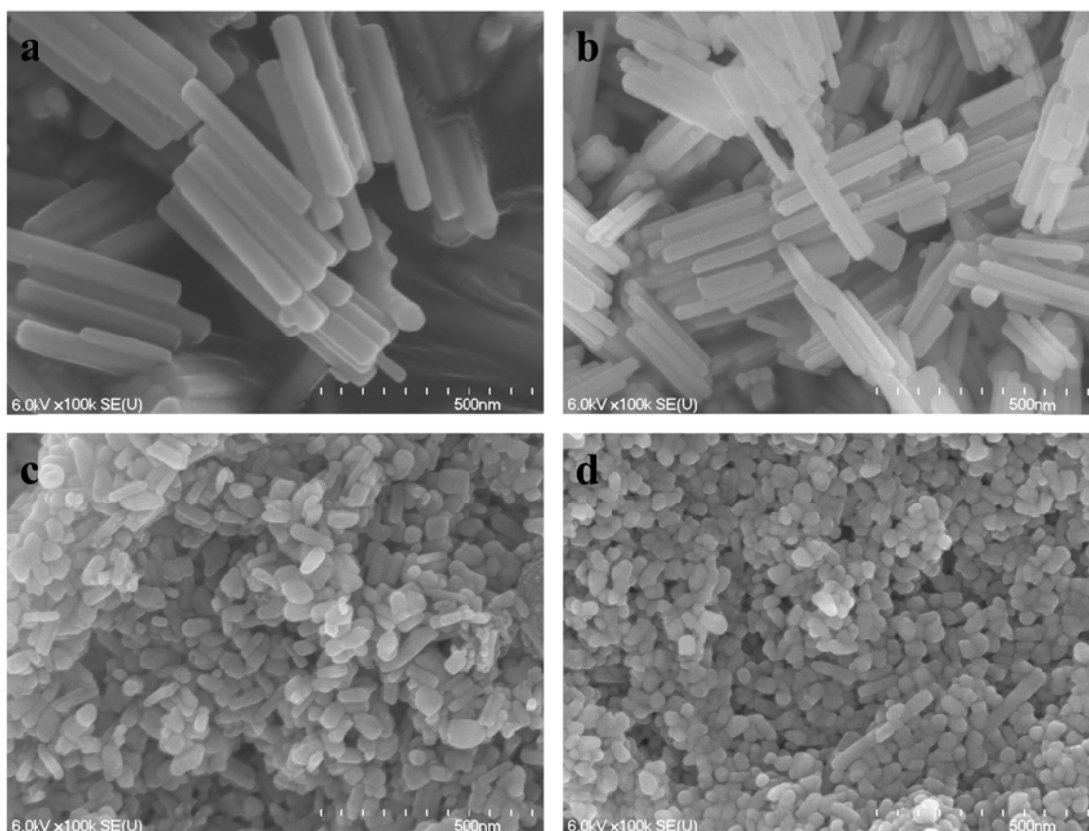


Figure II-2. SEM images of NaYF₄:Yb/Er (18/2 mol%) nanoparticles doped with (a) 30% Gd, (b) 45% Gd, (c) 60% Gd, and (d) 75% Gd. The scale bar for all the images is 200 nm.

For the synthesized LNPs to be used for biological studies, their dispersibility in water is a critical issue. We thus measured the effective hydrodynamic diameters (EHDs) of the synthesized LNPs in water by dynamic light scattering (DLS) to estimate the aggregation degree. NaYF₄:Yb/Er/Gd(18/2/30 mol%) and NaYF₄:Yb/Er/Gd(18/2/60 mol%) nanoparticles showed EHDs of 283 and 342 nm (Figure II-3), respectively, suggesting that 30% Gd doped nanorods are resistant to aggregation while 60% Gd doped ones are suffered from three-folds aggregation in comparison with the particle sizes measured in TEM images.

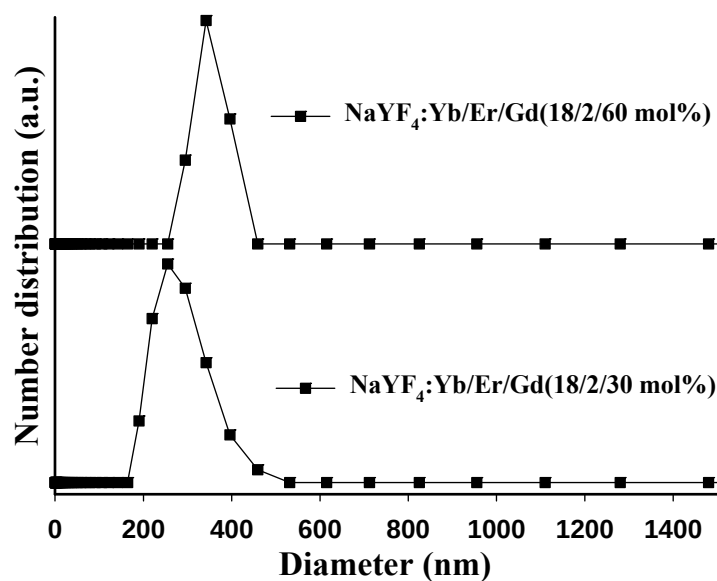


Figure II-3. Diameter distribution of 30% and 60% Gd doped NaYF₄:Yb/Er(18/2 mol%) nanoparticles in water, as determined by dynamic light scattering (DLS).

Under 980 nm excitation, upconversion luminescent spectra of 30% Gd NaYF₄:Yb/Er (18/2 mol%) nanoparticles in different solvents were recorded (Figure II-4).

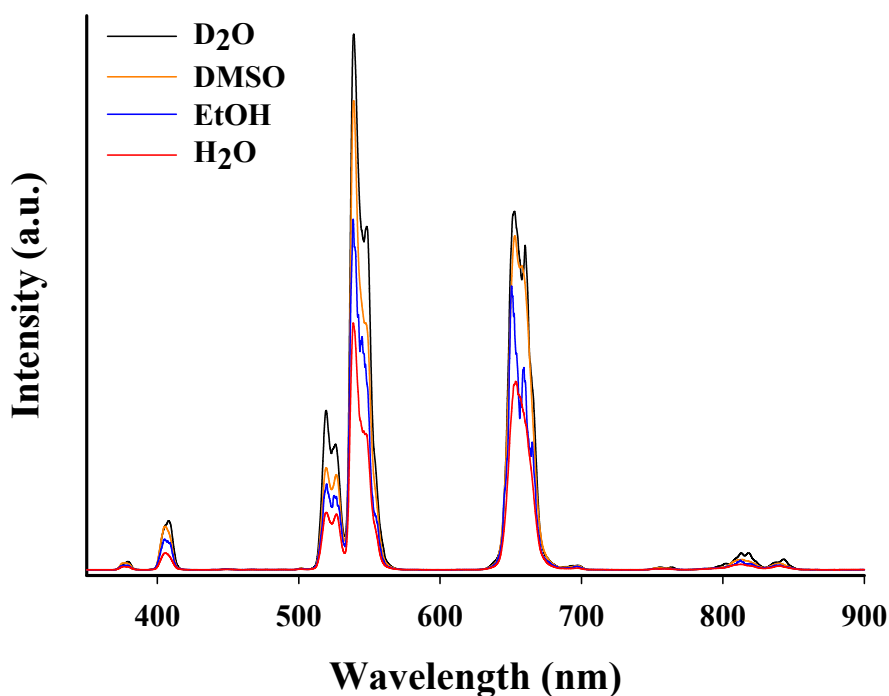


Figure II-4. Luminescent spectra of NaYF₄:Yb/Er/Gd (18/2/30 mol%) (1 mg/mL) nanoparticles in different solvents (D₂O, DMSO, EtOH and H₂O) with an excitation laser diode (150 mA, 980 nm) at room temperature.^[4]

Figure II-4 shows that NaYF₄:Yb/Er/Gd (18/2/30 mol%) nanoparticles gave green emission bands at 520 and 540 nm and red emission band at 655 in all tested solvents. The intensity decreases with the change of solvents in the order D₂O > DMSO > EtOH > H₂O. This solvent-induced luminescence reduction may be due to a solvent quenching, that is, a solvent-resonant multiphonon relaxation of excited states. In the energy ladder of Er³⁺ emission (Figure II-5), the energy levels, ³S_{3/2} and ⁴F_{9/2}, are responsible for the green (540 nm) and red (655 nm) emissions, and below them are some small step-gaps corresponding to solvent vibration energies. Water has a strong O-H stretching vibration (ca 3400 cm⁻¹) and a relatively strong combination-note vibration (ca 5200 cm⁻¹) of stretching and deformation, which correspond to the energy gaps of the non-radiative transitions [⁴S_{3/2}→⁴F_{9/2}] and [⁴F_{9/2}→⁴I_{11/2}], respectively. These non-radiative transitions might be resonated with the O-H vibrations to reduce the radiative transitions.

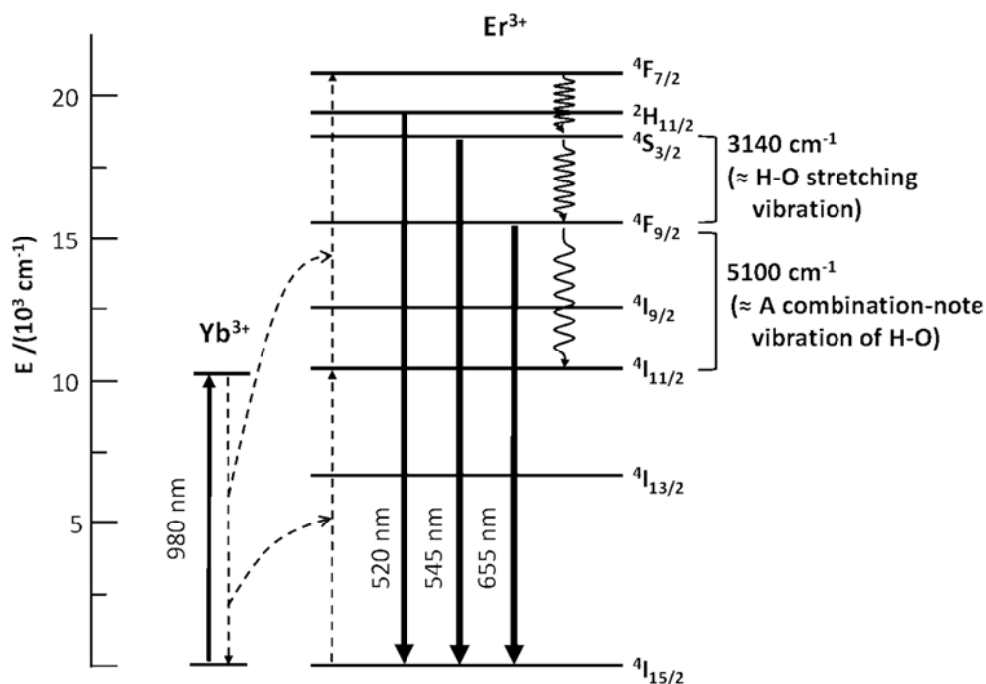


Figure II-5. An upconversion energy-transfer mechanism for the LNP emission and possible non-radiative transitions resonated with H₂O solvent.^[4]

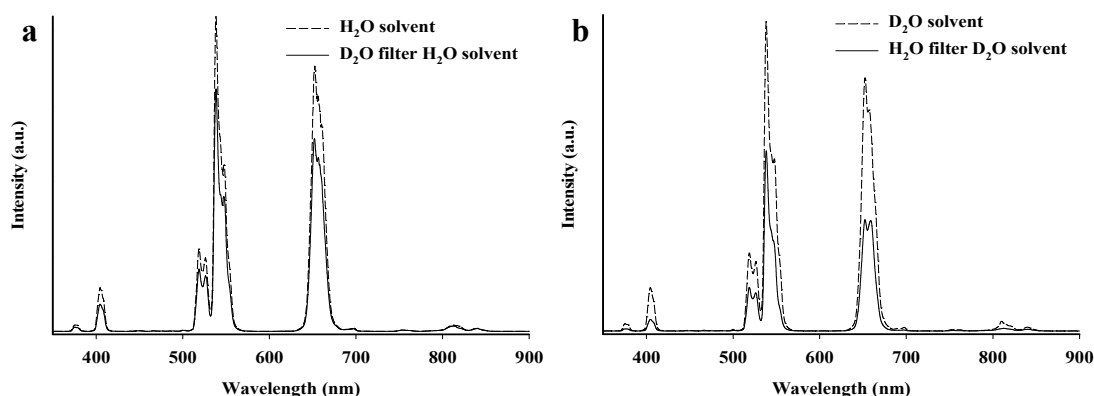


Figure II-6. A D₂O filter effect for the upconversion emission spectra of NaYF₄:Yb/Er/Gd (18/2/30 mol%) nanoparticles (0.4 mg/mL) in H₂O (a) and an H₂O filter effect for the same concentration of NaYF₄:Yb/Er/Gd (18/2/30 mol%) nanoparticles in D₂O (b). The emission spectra were measured with the laser excitation power of 400 mW (980 nm) and emission bandwidth of 3 nm.^[4]

Another possibility is that the incident near-infrared light at 980 nm was directly absorbed by a solvent and weakened. We assessed the latter possibility by using H₂O and D₂O filters (Figure II-6). The result obviously demonstrated that water absorbs the incident light to a great extent, i.e., while the D₂O filter barely affected the luminescence intensity (Figure II-6a), the H₂O filter drastically reduced the intensity by ca 42% at 540 nm and ca 56% at 655 nm (Figure II-6b). Therefore, the solvent effects in Figure II-4 are most likely due to a direct absorption of the incident light. The diminishment of the intensity at 540 nm was somewhat smaller than that observed in Figure II-4 (ca 54%). We suggest that the vibrations of water might have resonated with the non-radiative transitions to a small extent, causing the luminescence quench at the green emission by 12%. Overall, the solvent quenching of the LNP(Gd)-OA emission was smaller than expected and it was predicted that further coatings of LNPs would not dramatically increase the luminescence intensity.

Under 980 nm excitation, upconversion luminescent spectra of the samples with 0, 30, 45, 60 and 75% Gd doped upconversion nanoparticles were recorded at room temperature in water solution with concentration of 1 mg/mL for each, as shown in Figure II-7.

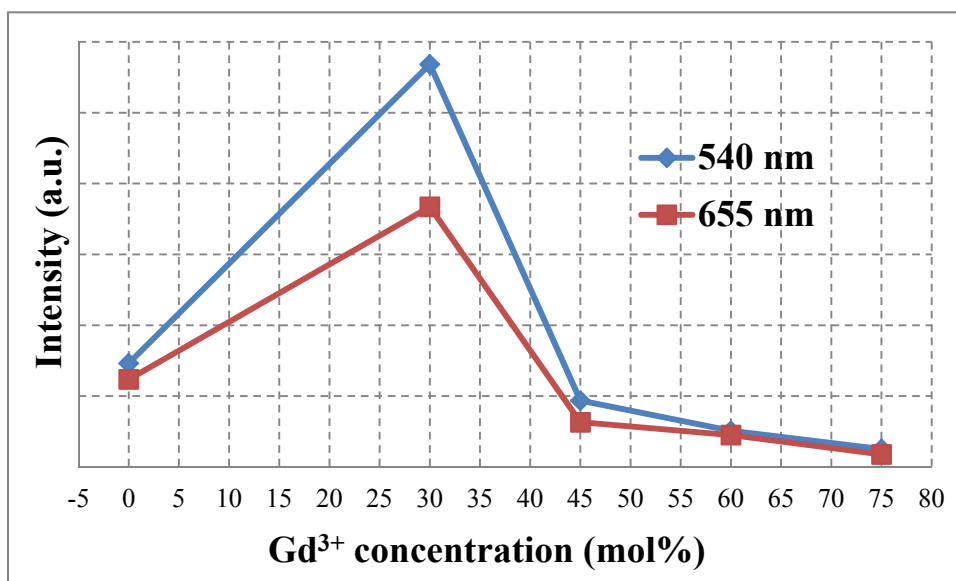


Figure II-7. Upconversion luminescent spectra of NaYF₄:Yb/Er with 0, 30, 45, 60 and 75% Gd under 980 nm excitation

The emission intensities at 540 and 655 nm as the function of Gd³⁺ concentration follow binomial distribution-like curves with the maximum intensities at 30% Gd³⁺. The increase of the intensity until 30% Gd³⁺ is likely to be due to the crystal phase transition from cubic to hexagonal (Figure II-8). The decrease of the intensity after 30% Gd³⁺ is ascribable to the size decrease of the hexagonal nanoparticles. We thus conclude that hexagonal crystal phase and larger size tend to increase the luminescent intensity of the upconversion nanoparticles.

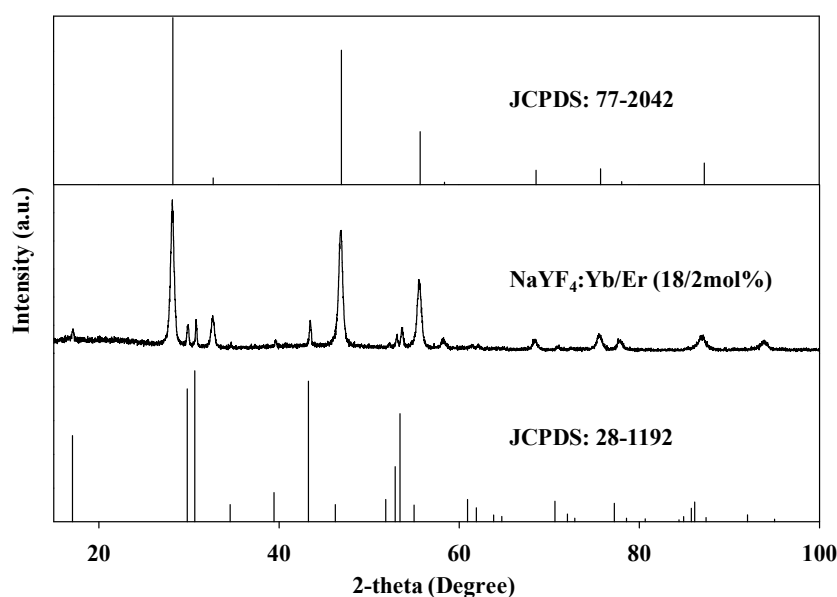


Figure II-8. XRD patterns of OA coated NaYF₄:Yb/Er (18/2 mol%)

Annealing effect was investigated on NaYF₄:Yb/Er/Gd(18/2/30 mol%) nanoparticles. After desiccation in vacuo, the nanoparticles were put into an oven and heated at 500 °C for 2 h. Figure II-9 shows that annealing effect could help increase the luminescent intensity at 655 nm while decrease the intensity at 540 nm. The annealing effect of NaYF₄:Yb/Er/Gd (18/2/30 mol%) nanoparticles didn't help improve the luminescent intensity at 410 and 540 nm which were resonated with the absorbance of PPIX.

The 30% Gd doped NaYF₄:Yb/Er nanoparticles showed the proper size and highest luminescent intensity which can be used in the further applications.

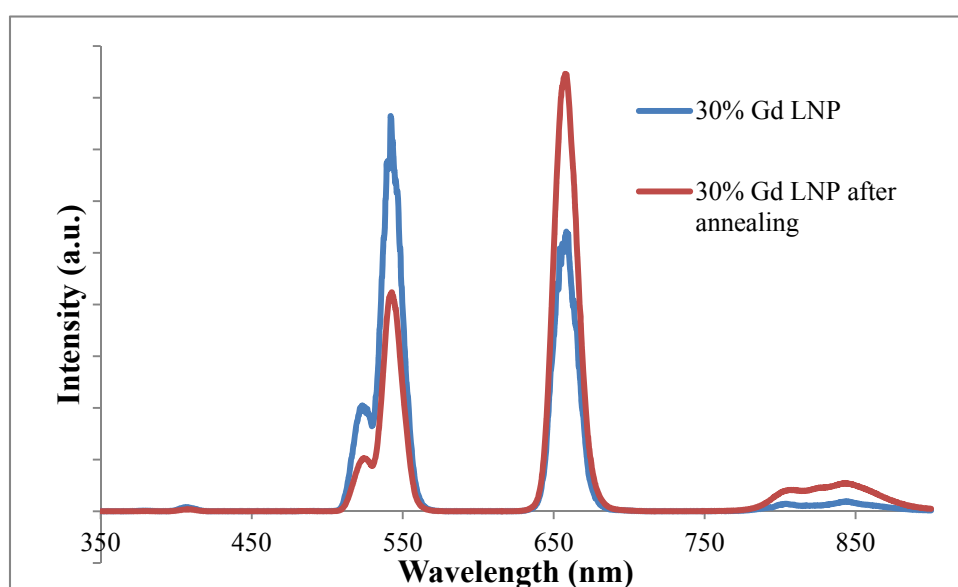


Figure II-9. Annealing effect on NaYF₄:Yb/Er/Gd(18/2/30 mol%) nanoparticles.

II-3 Boric acid scaffold hexagonal upconversion nanoparticles

Boron is a non-metallic element with low toxicity for biological use. Boron has an empty orbital which can be used as additive in upconversion nanoparticles. However, few reports were about boron in upconversion nanoparticles. Here boric acid as scaffold in upconversion nanoparticles was discussed.

In a typical synthesis, 2 mL of 0.5 mol/L RECl₃ (RE = Y/Yb/Er 80/18/2 mol%) aqueous

solution and 2 mmol of boric acid were dispersed into 40 mL of deionized water and magnetically stirred for 1 h, forming a chelated RE-B(OH)₃ complex. Then 16 mL of 0.5 mol/L NaF aqueous solution was added to the solution. After vigorous stirring for 1 h, the solution was transferred into a 100 mL of crystal glass reaction vessel, set for the autoclave, and heated at 160 °C for 18 h with stirring in the sealed stainless cylinder. After that it was cooled down slowly to room temperature. Subsequently, the suspension was centrifuged at 8000 r/min for 10 min. The resultant product was then washed thoroughly and dried in vacuo. The product was annealed at 500 °C for 2 h to improve the crystallinity.

The XRD pattern of the sample is presented in Figure II-10. All of the diffraction peaks are readily indexed to those of hexagonal NaYF₄:Yb/Er (JCPDS 28-1192).

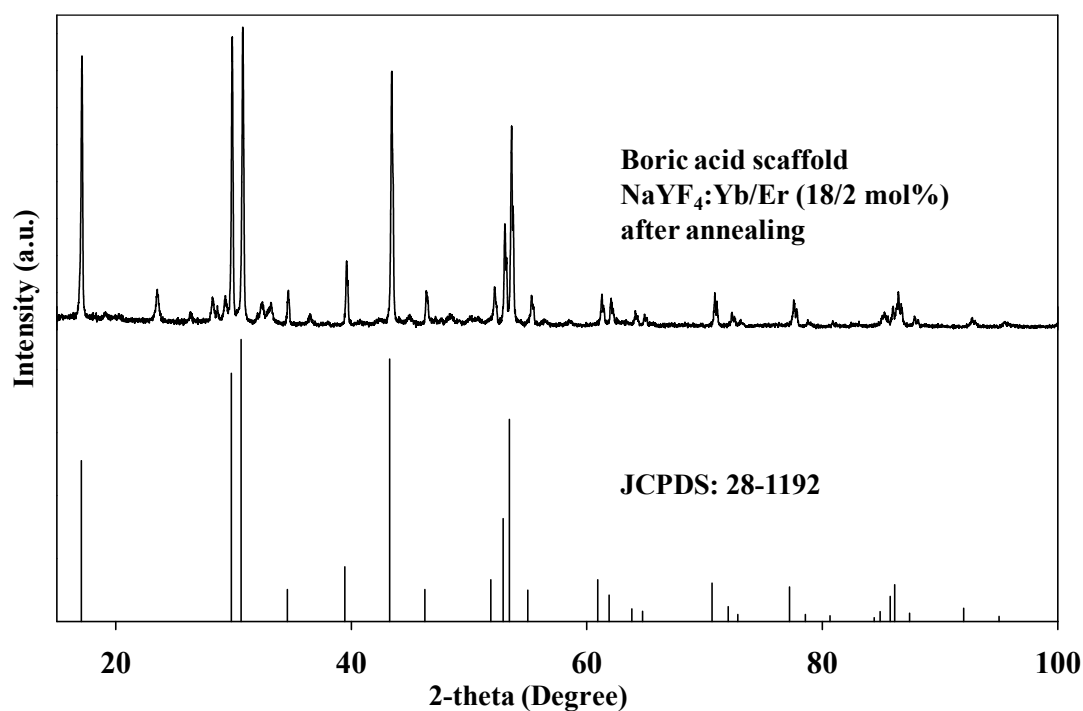


Figure II-10. XRD pattern of nanosized NaYF₄:Yb/Er after 2 h annealing

Under 980 nm excitation, upconversion luminescent spectra of the sample before and after 500 °C 2 h annealing was recorded at room temperature in water solution with concentration of 1 mg/mL for each, as shown in Figure II-11.

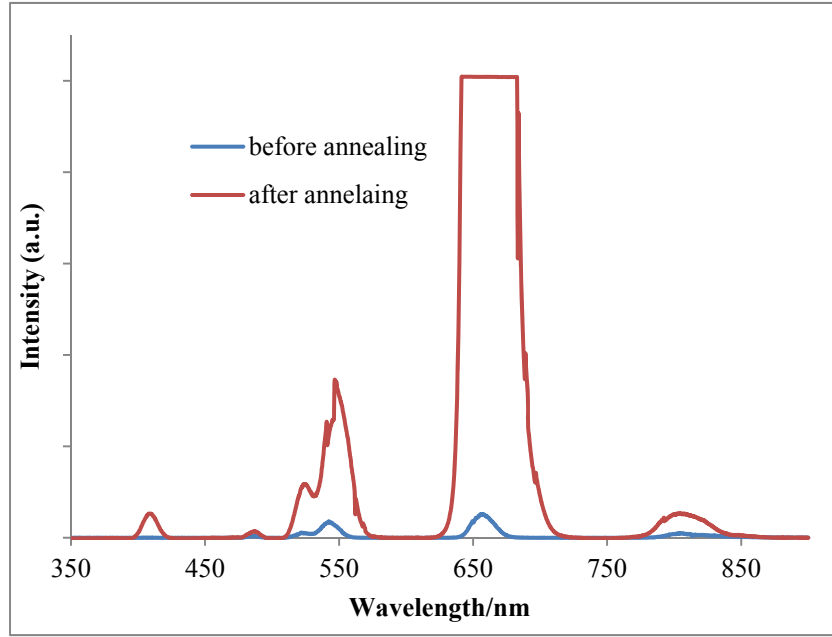


Figure II-11. Upconversion luminescent spectra of NaYF₄:Yb/Er under 980 nm excitation

After annealing, luminescent spectra of boric acid scaffold NaYF₄:Yb/Er upconversion nanoparticles improves obviously. New peak at 410 nm was observed, which is quite encouraged for the use in PDT experiment. Intensities at 540 and 655 nm increased significantly while intensity at 655 nm improved around 10 times after 500 °C 2 h annealing. From DLS result (Figure II-12), no obviously size change occurred before and after annealing.

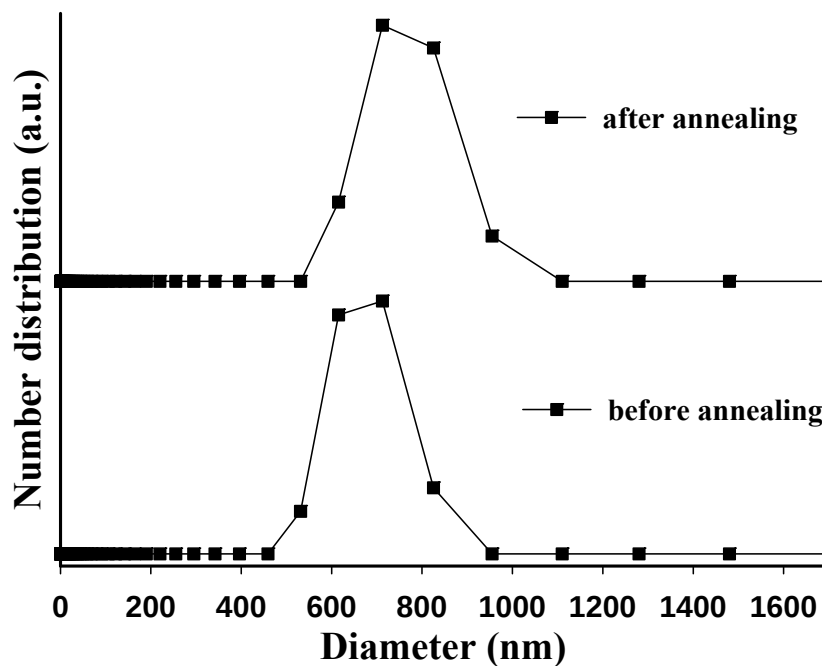


Figure II-12. Diameter distribution of boric acid scaffold $\text{NaYF}_4:\text{Yb/Er}(18/2 \text{ mol}\%)$ before and after annealing in water, determined by DLS.

In order to find the best condition of synthesizing the highest intensity of upconversion nanoparticles, boric acid amount, doped Gd amount, annealing temperature and NaF amount were investigated. The result was shown in Figure II-13.

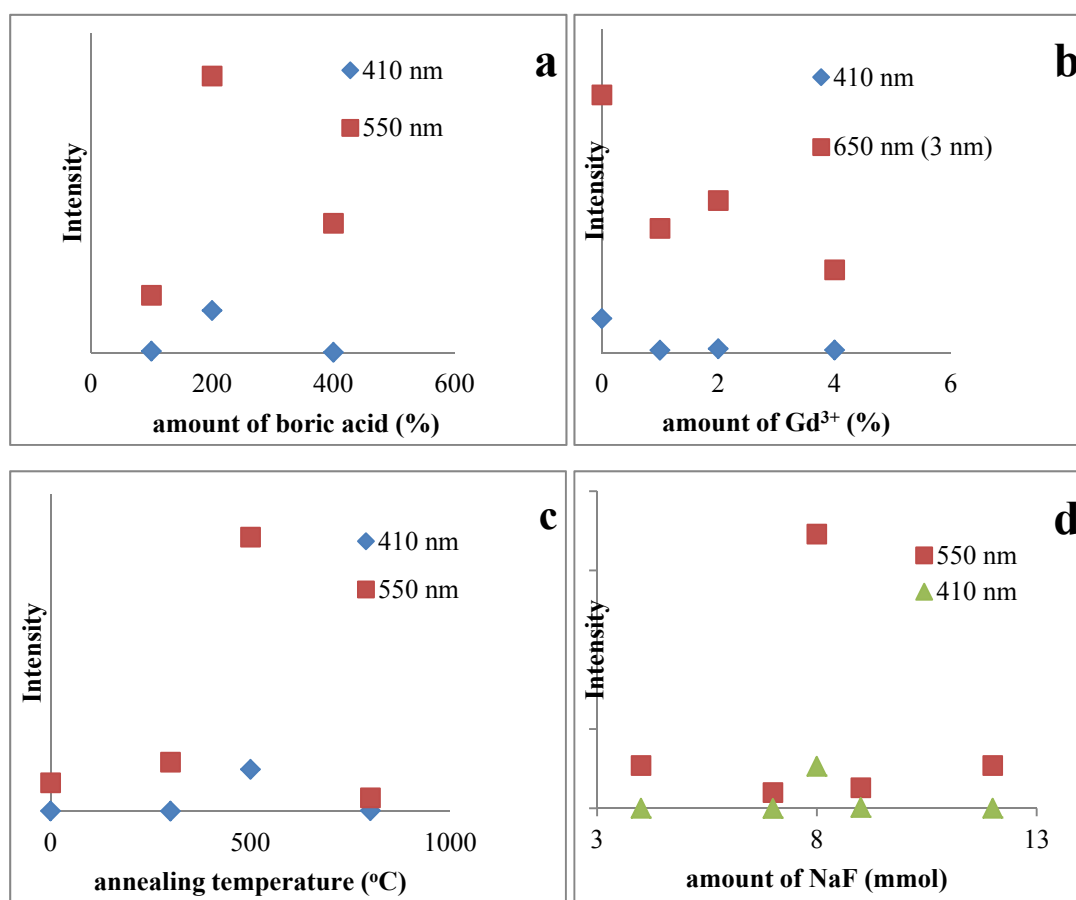


Figure II-13. Condition investigation of synthesizing high intensity of boric acid scaffold $\text{NaYF}_4:\text{Yb/Er}$ upconversion nanoparticles. (a) boric acid amount (100%, 200% and 400% comparing to RECl_3 amount) (b) amount of doped Gd (no doping, 1%, 2% and 4% comparing to RECl_3 amount) (c) annealing temperature (no annealing, 300, 500 and 800 °C) (d) NaF amount (4, 7, 8, 9 and 12 mmol).

From Figure II-13, 200% boric acid, no doped Gd, 500 °C annealing and 8 mmol NaF gave the best intensity of synthesizing boric acid scaffold $\text{NaYF}_4:\text{Yb/Er}$ upconversion nanoparticles, which

can be used in further applications.

II-4 EDTA scaffold hexagonal upconversion nanoparticles

Tm doped EDTA scaffold upconversion nanoparticles were reported by G. Wang et. al.^[3] that it showed strong luminescent intensity at 360 and 480 nm which seem that it can be used in PDT experiment. So in the later experiments, Tm doped EDTA scaffold upconversion nanoparticles were investigated.

Tm doped upconversion nanoparticles shows quite different luminescent spectra as Er ones.

In a typical synthesis, 2 mL of 0.5 mol/L RECl₃ (RE = Y/Yb/Tm 79/20/1 mol%) aqueous solution and 1 mmol of EDTA were dispersed into 40 mL of deionized water and magnetically stirred for 1 h, forming a chelated RE-EDTA complex. Then 16 mL of 0.5 mol/L NaF aqueous solution was added to the solution. After vigorous stirring for 1 h, the solution was transferred into a 100 mL of crystal glass reaction vessel, set for the autoclave, and heated at 160 °C for 18 h with stirring in the sealed stainless cylinder. After that it was cooled down slowly to room temperature. Subsequently, the suspension was centrifuged at 8000 r/min for 10 min. The resultant product was then washed thoroughly and dried in vacuo. The product was annealed at 500 °C for 2 h to improve the crystallinity.

The XRD pattern of the sample is presented in Figure II-14. All of the diffraction peaks are readily indexed to those of hexagonal YF₃:Yb/Tm (JCPDS 27-0814).

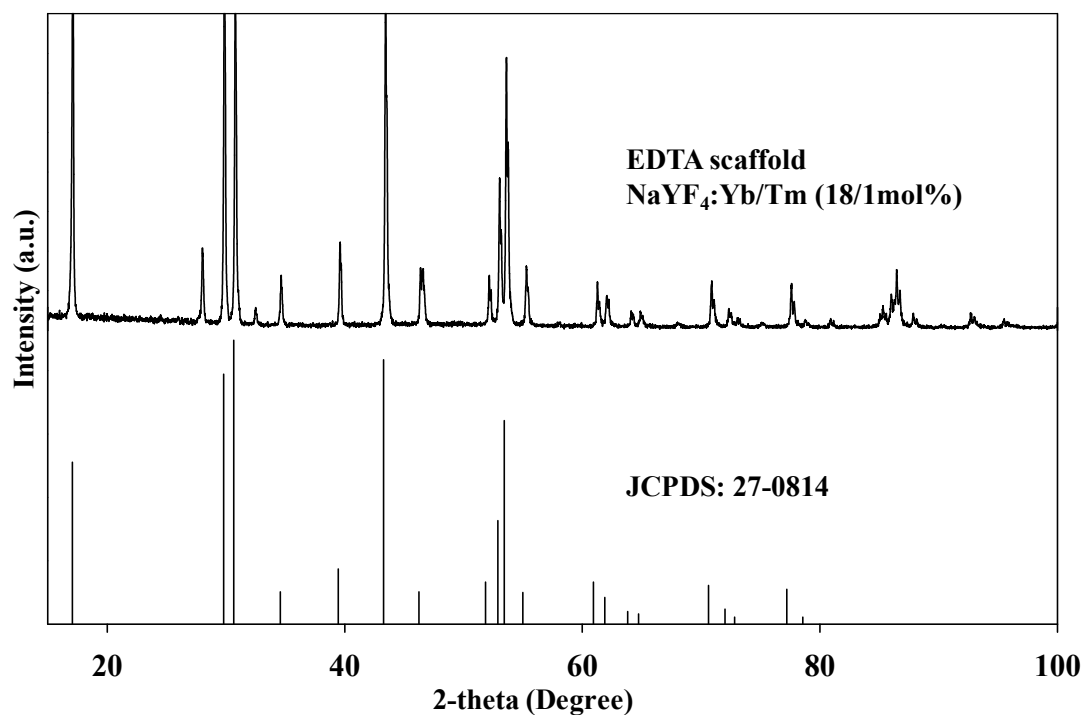


Figure II-14. XRD pattern of nanosized NaYF₄:Yb/Tm after 2 h annealing

Under 980 nm excitation, upconversion luminescent spectra of the sample before and after 500 °C 2 h annealing was recorded at room temperature in water solution with concentration of 1 mg/mL for each, as shown in Figure II-15.

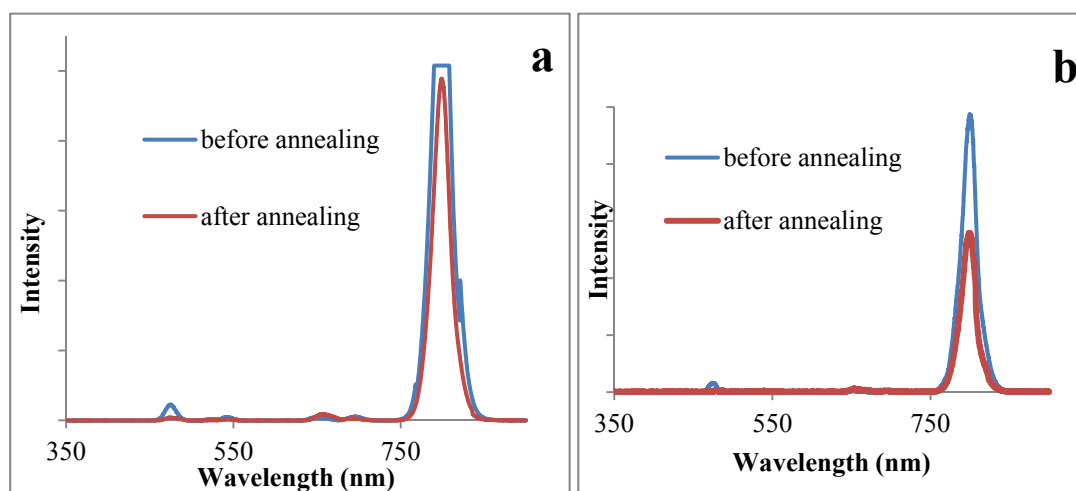


Figure II-15. Upconversion luminescent spectra of NaYF₄:Yb/Tm under 980 nm excitation with bandwidth (a) 10 nm and (b) 3 nm.

After annealing, luminescent spectra of EDTA scaffold NaYF₄:Yb/Tm upconversion nanoparticles decreased. Emission at 480 nm was disappeared and highest intensity at 800 nm decreased around 50% after annealing. Luminescent intensity at 360 nm was observed only when strong laser power was used. However that strong laser power can't be used in biological experiments. Tm doped EDTA scaffold upconversion nanoparticles are not suitable to be used in further application.

II-5 Other metal doped upconversion nanoparticles

According to the previous work, Gd doped upconversion nanoparticles were proved to give higher emission than other nanoparticles. In order to find some other efficient doping metal ions, Zn, Fe, Ni, Tm, V, Mo and Sm were doped in the synthesis system.

In a typical synthesis, 2 mL of 0.5 mol/L RECl₃ (RE = Y/Yb/Er 80/18/2 mol% and 2 mol% of ZnCl₂, FeCl₃, NiCl₂, TmCl₃, VCl₃, MoCl₃ or SmCl₃) aqueous solution and 2 mmol of boric acid were dispersed into 40 mL of deionized water and magnetically stirred for 1 h, forming a chelated RE-B(OH)₃ complex. Then 16 mL of 0.5 mol/L NaF aqueous solution was added to the solution. After vigorous stirring for 1 h, the solution was transferred into a 100 mL of crystal glass reaction vessel, set for the autoclave, and heated at 160 °C for 18 h with stirring in the sealed stainless cylinder. After that it was cooled down slowly to room temperature. Subsequently, the suspension was centrifuged at 8000 r/min for 10 min. The resultant product was then washed thoroughly and dried in vacuo. The product was annealed at 500 °C for 2 h to improve the crystallinity.

Under 980 nm excitation, upconversion luminescent spectra of the samples before and after 500 °C 2 h annealing were recorded at room temperature in water solution with concentration of 1 mg/mL for each, as shown in Figure II-13.

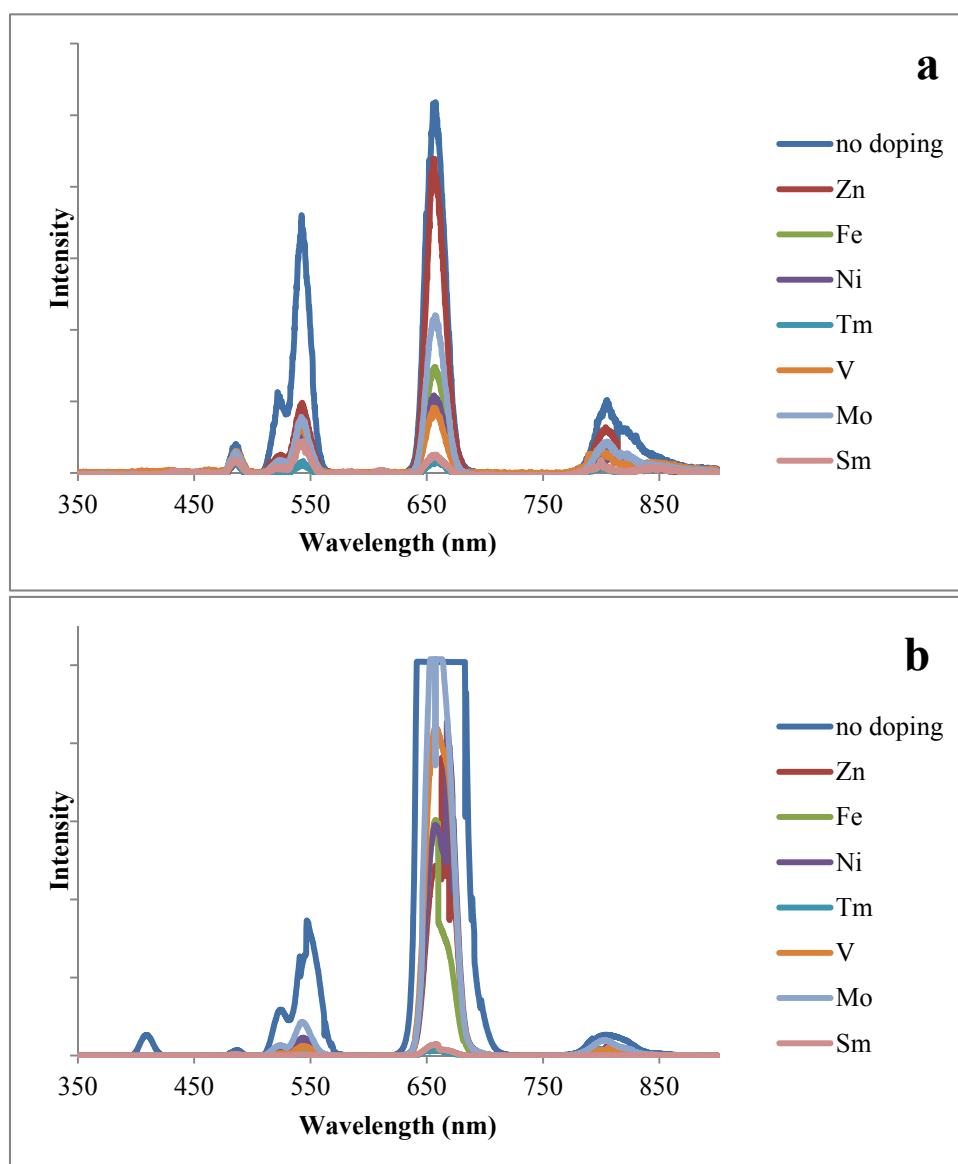


Figure II-16. Upconversion luminescent spectra of NaYF₄:Yb/Er/X (X = no doping, Zn, Fe, Ni, Tm, V, Mo and Sm) under 980 nm excitation (a) before and (b) after annealing.

It is obvious that these doped metal ions cannot help to improve the intensity of luminescent spectra of upconversion nanoparticles.

II-6 Summary

In this chapter, we are trying to synthesize upconversion nanoparticles with the highest luminescent intensity at 410, 540 and 655 nm.

By doping different amount of Gd, the luminescent intensities and nanoparticles sizes were decreased with the increase of Gd amount from 30% to 75%. All of them were hexagonal phase while nanoparticles without Gd doped were major cubic phase and minor hexagonal phase. OA coated $\text{NaYF}_4\text{:Yb/Er/Gd}$ (18/2/30 mol%) upconversion nanoparticles had significant strong luminescent intensity at 540 and 655 nm. For $\text{NaYF}_4\text{:Yb/Er/Gd}$ (18/2/30 mol%) upconversion nanoparticles, the intensity decreases with the change of solvents in the order $\text{D}_2\text{O} > \text{DMSO} > \text{EtOH} > \text{H}_2\text{O}$. Annealing effect for $\text{NaYF}_4\text{:Yb/Er/Gd}$ (18/2/30 mol%) upconversion nanoparticles was negative.

The intensity of hexagonal phase boric acid doped $\text{NaYF}_4\text{:Yb/Er}$ (18/2 mol%) nanoparticles was improved obviously after annealing at 500 °C for 2 h and new peak at 410 nm was observed which was quite suitable to be resonated with the absorbance of PPIX. The reaction conditions were investigated. 200% boric acid doped, no doping Gd, 500 °C annealing and 8 mmol NaF gave the best intensity of synthesizing boric acid doped $\text{NaYF}_4\text{:Yb/Er}$ upconversion nanoparticles.

The luminescent spectra of EDTA scaffold $\text{NaYF}_4\text{:Yb/Tm}$ (20/1 mol%) nanoparticles were quite different from $\text{NaYF}_4\text{:Yb/Er}$ (18/2) nanoparticles. Significant strong peak was observed at 800 nm and small peak at 480 nm. Annealing effect was also negative for $\text{NaYF}_4\text{:Yb/Tm}$ (20/1 mol%) nanoparticles.

Zn, Fe, Ni, Tm, V, Mo and Sm ions were doped into upconversion nanoparticles to investigate the influence of dopants. Annealing effect was positive to these nanoparticles while metal ion dopants can't help to improve the luminescent intensity of upconversion nanoparticles.

$\text{NaYF}_4\text{:Yb/Er/Gd}$ (18/2/30 mol%) nanoparticles showed great emission intensity at 540 and 655 nm with proper size while boric acid scaffold upconversion nanoparticles showed obvious emission intensity at 410, 540 and 655 nm after annealing at 500 °C for 2 h. Both of their emission wavelengths were resonated with the absorbance of PPIX and can be used in further application of PDT experiment.

Reference

- [1] F. Wang, Y. Han, C. S. Lim, Y. Lu, J. Wang, J. Xu, H. Chen, C. Zhang, M. Hong, X.

Liu, Simultaneous phase and size control of upconversion nanocrystals through lanthanide doping, *Nature* **2010**, 463, 1061-1065. DOI: 10.1038/nature08777.

[2] F. Chen, W. Bu, S. Zhang, X. Liu, J. Liu, H. Xing, Q. Xiao, L. Zhou, W. Peng, L. Wang, J. Shi, Positive and negative lattice shielding effects co-existing in Gd (III) ion doped bifunctional upconversion nanoprobe, *Adv. Funct. Mater.* **2011**, 21, 4285-4294. DOI: 10.1002/adfm.201101663.

[3] G. Wang, W. Qin, L. Wang, G. Wei, P. Zhu, D. Zhang, F. Ding, Enhanced ultraviolet upconversion in $\text{YF}_3\text{:Yb}^{3+}/\text{Tm}^{3+}$ nanocrystals, *J. Rare Earth* **2009**, 27, 330-333. DOI: 10.1016/S1002-0721(08)60245-8.

[4] Y. Liu, T. Kobayashi, M. Iizuka, T. Tanaka, I. Sotokawa, A. Shimoyama, Y. Murayama, E. Otsuji, S. Ogura, H. Yuasa, Sugar-attached upconversion lanthanide nanoparticles: a novel tool for high-throughput lectin assay, *Bioorg. Med. Chem.* **2013**, 21, 2832-2842. DOI: 10.1016/j.bmc.2013.04.008.

Chapter III Sugar-attached upconversion lanthanide nanoparticles

III-1 Introduction

With proper upconversion nanoparticles in hand, next sugar-attached upconversion nanoparticles should be studied.

Surface modification is one of the most important fields in upconversion lanthanide nanoparticles research. The ideal surface modification can help upconversion lanthanide nanoparticles to obtain homogeneous dispersibility, stability in aqueous solution, improvement of luminescent intensity, and platform for the attachment of biomolecules. For example, the studies of bioimaging *in vivo*,^[1-4] analyte detection *in vitro*,^[5] DNA vaccines,^[6] and etc. all exploited biomolecule-attached LNPs.

Recently, the most commonly used surface modifications were carboxyl group and amine group. In the experiments of this thesis, polyethylenimine (PEI) and oleic acid (OA) provide an amino and carboxyl (after oxidation of the ethylenic bond in OA) functional groups, respectively, on the surface of UCLNPs, permitting the attachment of biomolecules through covalent linkers.

In this chapter, we focused on the attachment of monosaccharides to UCLNPs using monosaccharides as special ligands of lectins on the surface of cells. We thus attached mannose on PEI and OA-coated UCLNPs with or without Gd^{3+} dopant because mannose is reported as a special ligand of the mannose receptor of cancer-associated macrophage as it is mentioned in Introduction Section. We studied the effect of the combination of additives on UCLNPs, solvents, linkers, and addition of mannose on luminescent intensity. Because galactose-binding lectins are often expressed on cancer cells, a galactose-attached UCLNP was also synthesized.

Lectins are defined as a kind of proteins which agglutinate cells and are sugar specific. With their unique properties, lectins can be used for cell identification and separation; detection, isolation, and structural studies of glycoproteins; investigation of carbohydrates on cells and subcellular organelles; mapping of neuronal pathways; mitogenic stimulation of lymphocytes; purging of bone marrow for transplantation; selection of lectin-resistant mutants; and

studies of glycoprotein biosynthesis.^[7] In our experiments, we mainly use the lectins of their sugar-specific ability.

With monosaccharide-attached LNPs in hand, we tested the combination of monosaccharides and different lectins using lectin aggregation assay.

Concanavalin A (ConA) is a lectin originally extracted from the jack-bean, *Canavalia ensiformis*. It binds specifically to certain structures found in various sugars, glycoproteins, and glycolipids, mainly internal and nonreducing terminal α -D-mannosyl and α -D-glucosyl groups. ConA is a homotetramer: each sub-unit (26.5KDa, 235 amino-acids, heavily glycosylated) binds a metallic atom (usually Mn^{2+} and a Ca^{2+}). ConA was used in lectin aggregation assay of mannose-attached LNPs.

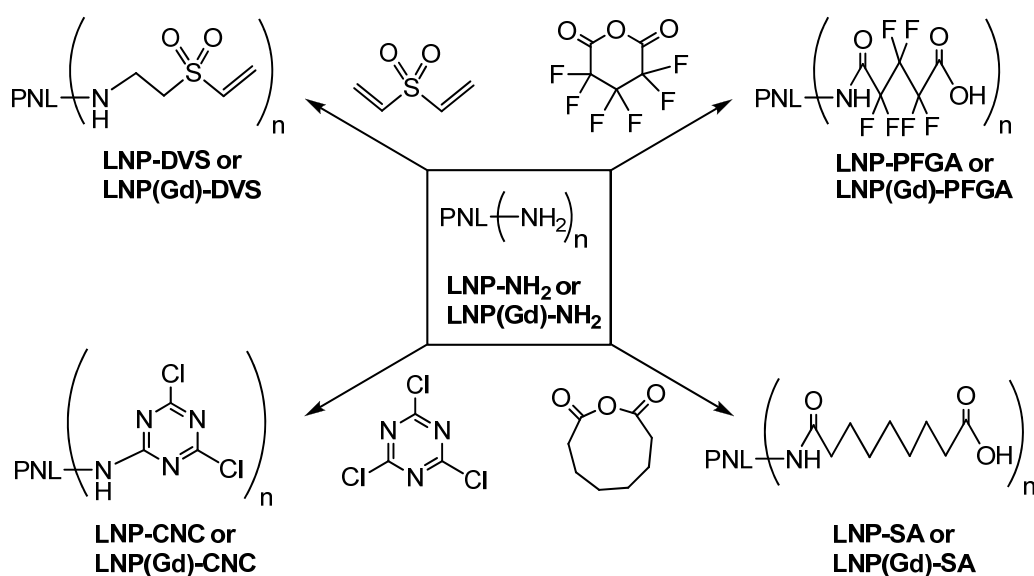
For galactose-attached LNPs, another lectin peanut agglutinin (PNA), a galactose-recognizing lectin, was used in lectin aggregation assay.

Finally, we used monosaccharide-attached LNPs in macrophage aggregation assay.

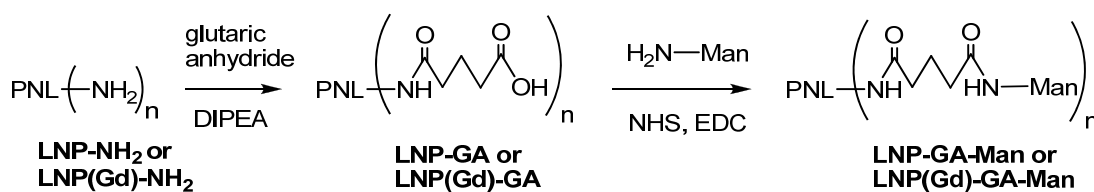
III-2 Attachment of sugar to upconversion nanoparticles

III-2-1 Attachment of sugar to PEI coated UCLNPs

PEI coated UCLNPs has amine group on the surface. In order to attach mannose to the surface of UCLNPs, linker reagents were investigated. In the previous studies of Mr. Kobayashi (a master student in Yuasa Lab.), five linkers, divinyl sulfone (DVS), cyanuric chloride (CNC), glutaric anhydride (GA), perfluoroglutaric anhydride (PFGA), and suberic anhydride (SA), were chosen as linker reagents. The changes in the emission intensity (I/I_0) at 655 nm by the linker attachment to PEI coated UCLNPs (2.5 mg/mL) for DVS/CNC/GA/PFGA/SA were 0.11/0.63/0.83/1.08/0.73. Though the highest intensity was obtained with PFGA, the reagent is quite expensive and thus we concluded that the choice of GA as linker is compromisable for PEI coated UCLNPs.



Scheme III-1. Attachment of various linkers to PEI coated UCLNPs: all the reaction were carried out in DMF.^[13]



Scheme III-2. The synthetic scheme of mannose attached UCLNPs from PEI coated UCLNPs (n: an unspecific number, DIPEA: *N,N'*-diisopropylethylamine).^[13]

Mannose-coated NaYF₄:Yb/Er (10/2 mol%), LNP-GA-Man, and mannose-coated NaYF₄:Yb/Er/Gd (10/2/30 mol%), LNP(Gd)-GA-Man, were synthesized from the corresponding LNPs and H₂N-Man using glutaric anhydride as a linker reagent and *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as coupling reagents (Scheme II-2). These nanoparticles were dispersible in water, which is a preferable property for biological use. Interestingly, mannose attachment caused size-reductions for both LNP-NH₂ and LNP(Gd)-NH₂, the diameters being 10-15 nm (Figure III-1b and 1d). A possible explanation that the mechanical stirring of LNPs would have caused abrasion was ruled out, because the TEM image of LNP-NH₂ after 24 h stirring in water showed no size-reduction. Perhaps, the reagents used for the linker and sugar attachment caused a chemical erosion of the lanthanide crystals. Chemical erosion might also explain the morphological change of LNP(Gd)-GA-Man from nanocubes to nanoballs after the mannose attachment (Figure III-1d).

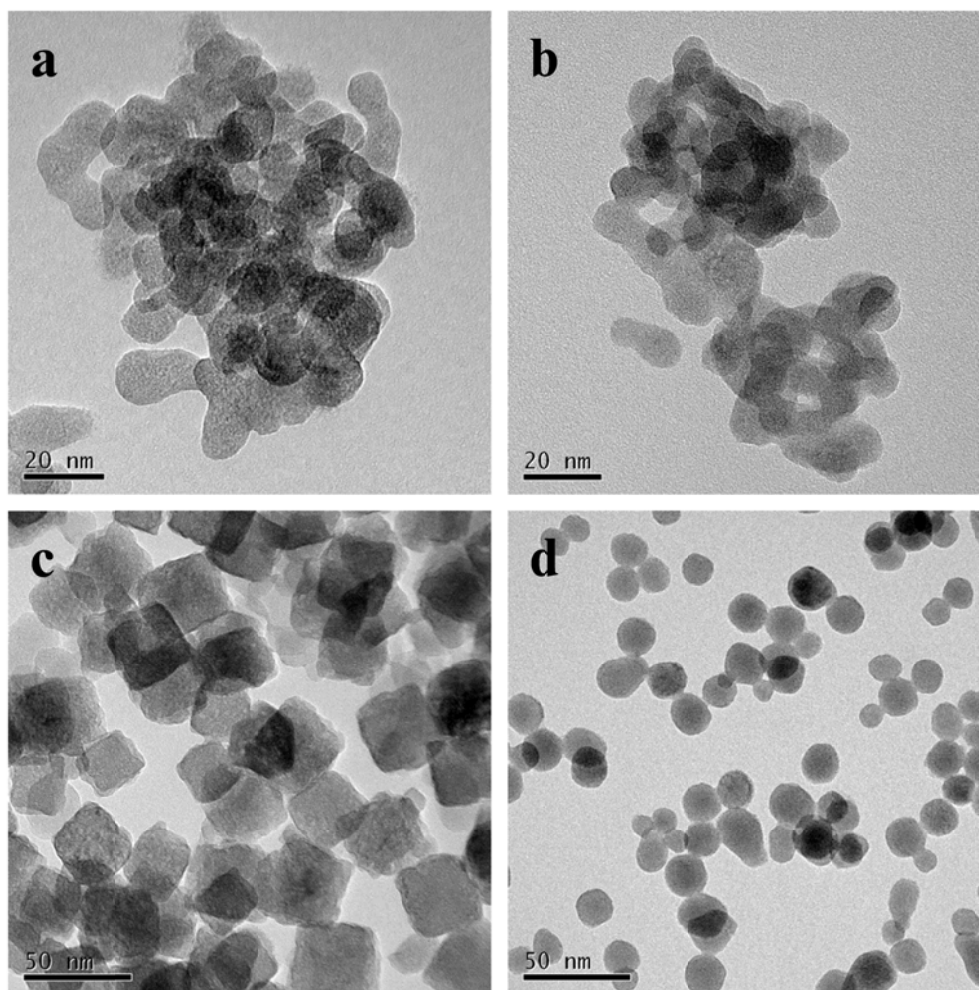


Figure III-1. TEM images of (a) LNP-NH₂, (b) LNP-GA-Man, (c) LNP(Gd)-NH₂, and (d) LNP(Gd)-GA-Man. The scale bars designate 20 nm for a and b and 50 nm for c and d.^[13]

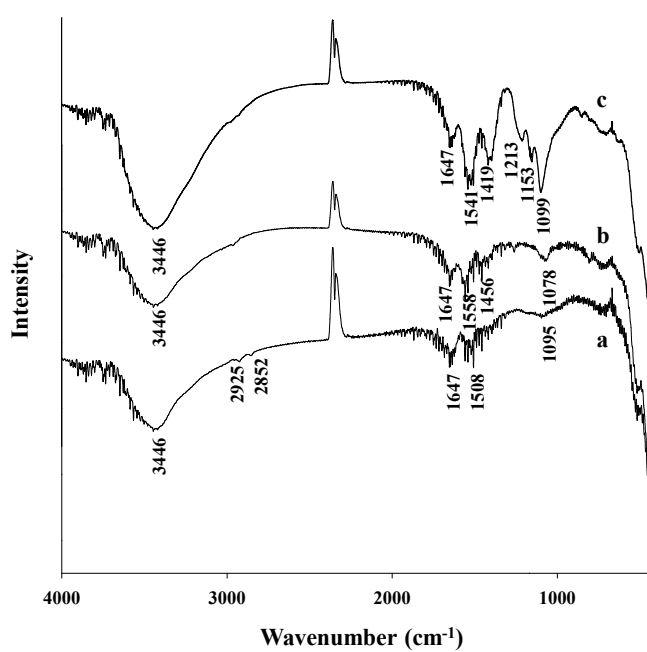


Figure III-2. FT-IR spectra of the upconversion nanoparticles: (a) LNP-NH₂, (b) LNP-GA, and (c) LNP-GA-Man.^[13]

Monosaccharide attachment to LNPs was proved by FT-IR spectra (Figure III-2) and phenolsulfuric acid assay. All the PEI-coated LNP and its derivatives (Figure III-2a-2c) exhibited a broad band at 3446 cm⁻¹, corresponding to an N-H or O-H stretching vibration. The absorptions at 1647 cm⁻¹ for three samples can be assigned to N-H bending vibration. There was no drastic variation between spectra a and b, but spectrum c showed significant appearance of peaks at 1541, 1419, 1213, 1153, and 1099 cm⁻¹. The peak at 1541 cm⁻¹ corresponds to a typical absorbance of an N-H bending vibration for amides. The peaks from 1099 to 1213 cm⁻¹ are ascribable to the C-O stretching and C-O-C asymmetric stretching vibrations in mannose. Thus a significant amount of mannose seemed to be attached on the LNP-GA-Man surface. Phenolsulfuric acid assay showed that LNP-GA-Man and LNP(Gd)-GA-Man have 47±3 and 4.0 ±1.2 μmol/g mannose molecules, respectively. The small mannose-loading on LNP(Gd)-GA-Man might be due to the large degree of chemical erosion as observed in Figure III-1d.

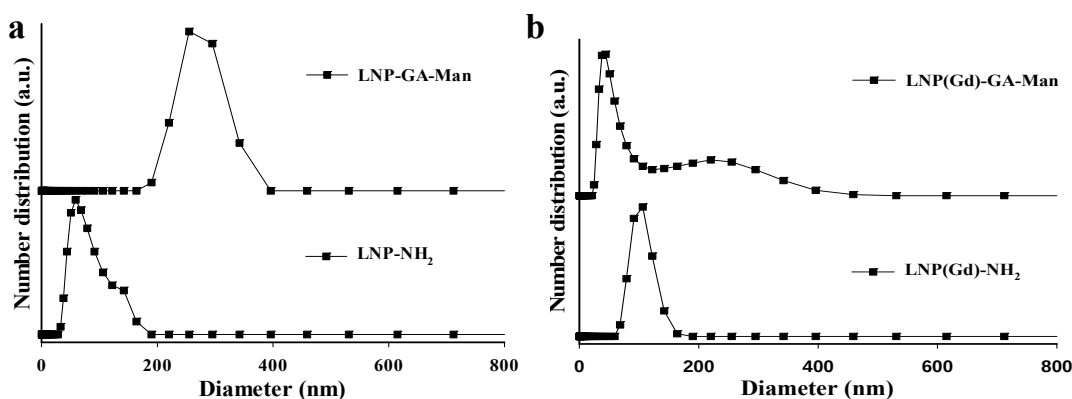


Figure III-3. Diameter distribution of (a) LNP-NH₂ and LNP-GA-Man and (b) LNP(Gd)-NH₂ and LNP(Gd)-GA-Man in water, determined by dynamic light scattering (DLS).

For the synthesized UCLNPs to be used for biological studies, their dispersibility in water is critical issue. We thus measured the effective hydrodynamic diameters (EHDs) of the synthesized UCLNPs in water by dynamic light scattering (DLS) to estimate the aggregatio degree.

LNP-NH₂ showed a 74 nm EHD (Figure III-3a), which is almost the same size as an aggregate observed in TEM image (Figure III-1a). However, EHD of LNP-GA-Man apparently increased to ca 200 nm by the mannose addition (Figure III-3a), perhaps owing to the reduced number of the mutually repulsive ammonium ions that might contribute to the dispersibility of LNP-NH₂. EHDs of Gd-doped UCLNPs, LNP(Gd)-NH₂ and LNP(Gd)-GA-Man, were 115 and 69 nm (Figure III-3b), three and six-folds larger than those in TEM images, respectively, showing again that the addition of mannose causes aggregation.

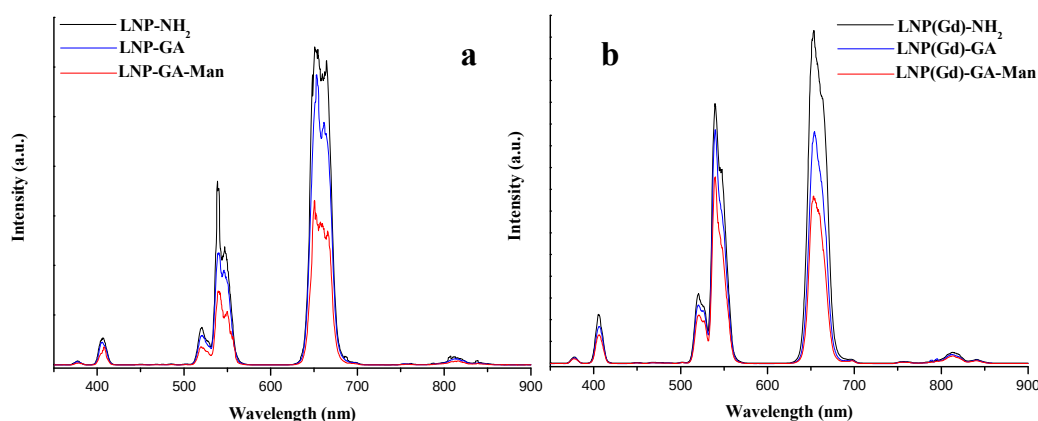
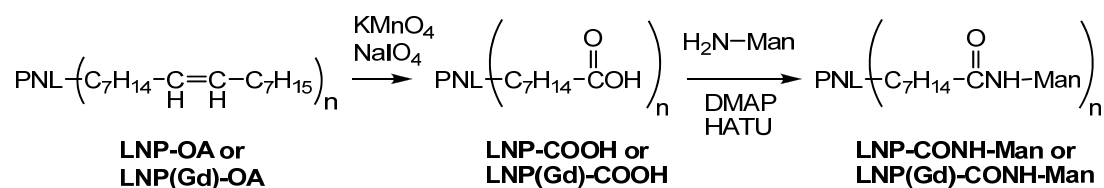


Figure III-4. The upconversion emission spectra of UCLNPs in water before and after mannose unit attachment: (a) LNP-NH₂ series (1 mg/mL) and (b) LNP(Gd)-NH₂ series (1 mg/mL). The emission spectra were measured with the laser excitaiton power of 400 mW (980 nm) and emission bandwidth of 3 nm.^[13]

The effect of the linker and mannose attachments on the luminescence spectra of UCLNPs were studied. In the case of LNP-NH₂ and LNP(Gd)-NH₂, where PEI was used as the coating agent, the luminescence intensities gradually decreased as the modification proceeded (Figure III-4a,b). For LNP-NH₂, the signal reduction was greater at 540 nm than at 655 nm, and vice versa for LNP(Gd)-NH₂. These observations are likely due to the difference in the favored quenching modes between the green-light quenching [⁴S_{3/2}→⁴F_{9/2}] ($\approx 3140\text{ cm}^{-1}$) caused by the stretching vibration of O-H groups and the red-light quenching [⁴F_{9/2}→⁴I_{11/2}] ($\approx 5100\text{ cm}^{-1}$) caused by the combination note vibration.

III-2-2 Attachment of sugar to OA coated UCLNPs



Scheme II-3. The synthetic scheme of LNP-CONH-Man or LNP(Gd)-CONH-Man from LNP-OA or LNP(Gd)-OA (n: an unspecific number).^[13]

In the case of OA-coated UCLNPs, they were oxidized with Lemieux-von Rudloff reagent as reported by Z. Chen, et al.,^[8] in order to generate a carboxyl functionality, which enables the coupling with amino-functionalized glycosides (Scheme II-3). The carboxylated UCLNPs, LNP-COOH and LNP(Gd)-COOH, were then converted into mannose-coated NaYF₄:Yb/Er (18/2 mol%), LNP-CONH-Man, and mannose-coated NaYF₄:Yb/Er/Gd (18/2/30 mol%), LNP(Gd)-CONH-Man, by using 4-dimethylaminopyridine (DMAP) and *o*-(7-azabenzotriazol-yl-1)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) as the coupling reagents. These mannose-coated UCLNPs were water dispersible. TEM images show that the size and morphology of LNP-OA and LNP(Gd)-OA hardly changed throughout the mannose modification processes (Figure III-5a→5b and 5c→5d).

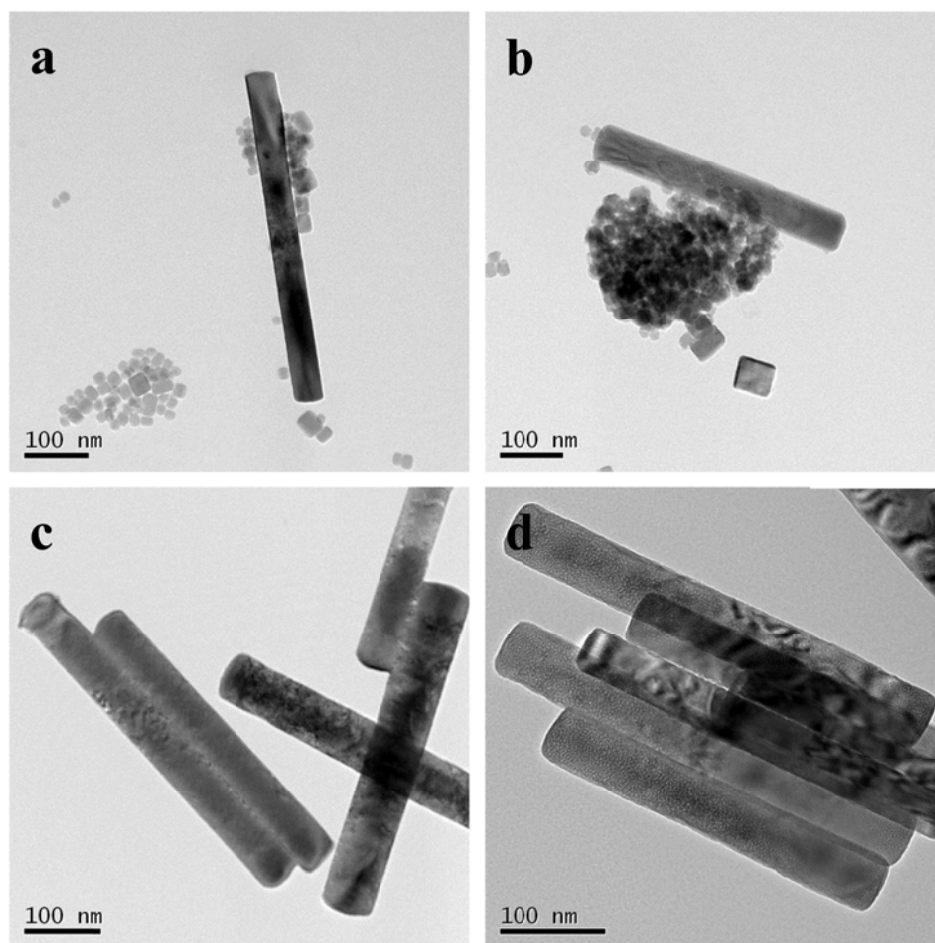


Figure III-5. TEM images of (a) LNP-OA, (b) LNP-CONH-Man, (c) LNP(Gd)-OA, and (d) LNP(Gd)-CONH-Man. The scale bars designate 100 nm for a-d.^[13]

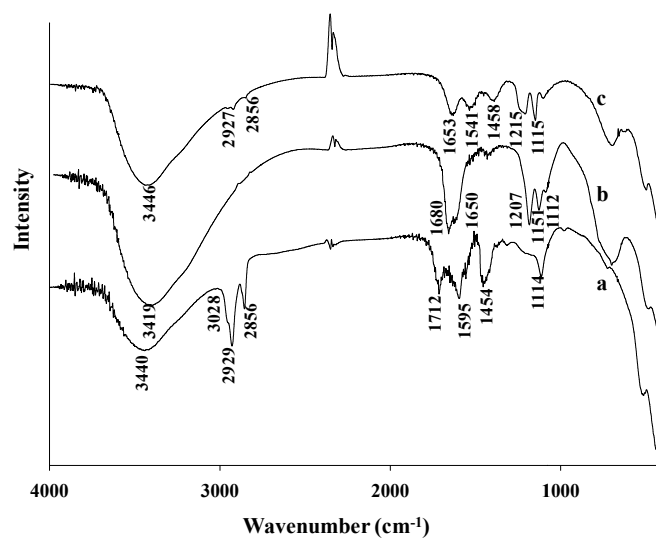


Figure III-6. FT-IR spectra of the upconversion nanoparticles: (a) LNP-OA, (b) LNP-COOH, and (c) LNP-CONH-Man.^[13]

Confirmation of the carboxyl group and mannose on the LNPs was made by FT-IR spectra (Figure III-6) and phenol-sulfuric acid assay. All the OA-coated LNP and its derivatives (Figure III-6a-6c) exhibited broad bands at 3419-3446 cm^{-1} , corresponding to an O-H or N-H stretching vibration. The peaks at 2929 and 2856 cm^{-1} in spectrum a are respectively assigned to the asymmetric and symmetric stretching vibrations of the methylene $-\text{CH}_2-$ group in OA. A peak at 3028 cm^{-1} could be attributed to $=\text{C}-\text{H}$ stretching vibration. The peak at 1712 cm^{-1} corresponds to the $\text{C}=\text{O}$ stretching vibration of the carboxyl group. The peaks at 1595, 1454, and 1114 cm^{-1} are suggested to originate from the asymmetric stretching vibration of carboxylate anions, deformation vibration of $-\text{CH}_2-$, and skeletal vibration of CH_3 , respectively (Figure III-6a). Bands at 1680 and 1650 cm^{-1} in spectrum b correspond to the $\text{C}=\text{O}$ stretching vibration for the carboxyl groups of LNP-COOH. The peaks between 1207 and 1112 cm^{-1} attributed to C-O-C stretching vibrations suggest the formation of anhydrides in the oxidation of LNP-OA (Figure III-6b). For LNP-CONH-Man, the peaks at 1653 and 1541 cm^{-1} are attributable to $\text{C}=\text{O}$ stretching and N-C=O symmetric stretching vibrations, respectively. Though the peaks at 1215 and 1115 cm^{-1} correspond to the C-O stretching vibrations of mannose residue, these are not decisive owing to the overlaps with the peaks for LNP-COOH (Figure III-6c). The existence of mannose on the surface of LNP(Gd)-CONH-Man was also confirmed by a lectin aggregation as described in the later section of this paper. The phenol-sulfuric acid assay for LNP-CONH-Man and LNP(Gd)-CONH-Man showed that these LNPs include 56 ± 5 and 93 ± 0.5 $\mu\text{mol/g}$ of mannose. The larger mannose loading on LNP(Gd)-CONH-Man relative to LNP-CONH-Man is consistent with the size difference of the two LNPs. Although we were able to improve the mannose loading up to 196 ± 40 $\mu\text{mol/g}$ by using sonication, the long operation time was harmful for the sonicator and thus has never been examined again.

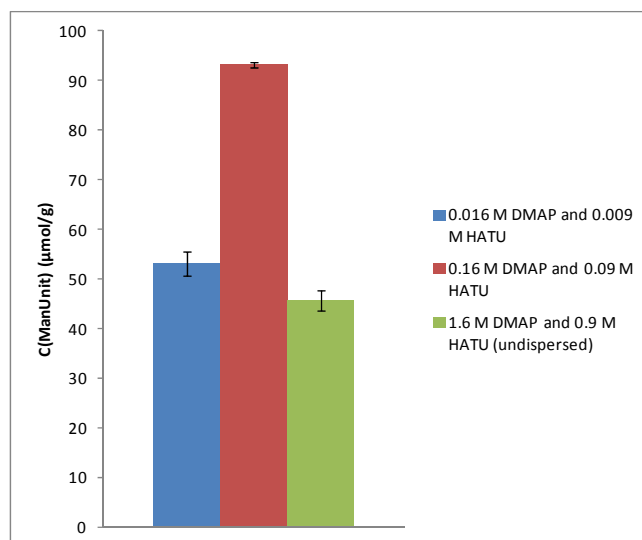


Figure III-7. The phenol sulfuric acid assay results for mannose-coated LNPs using the different concentrations of DMAP/HATU as coupling reagents.

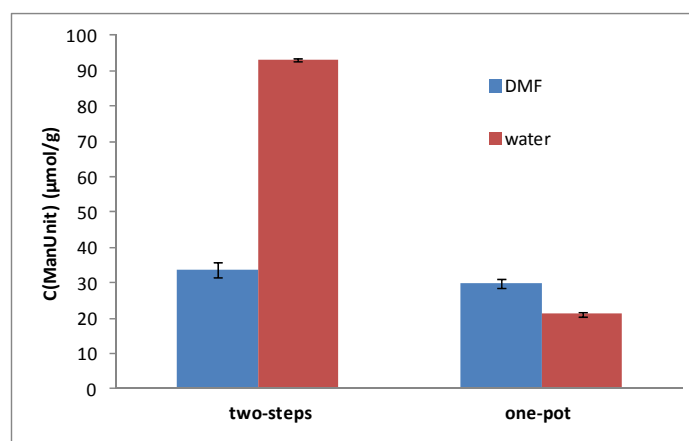


Figure III-8. The phenol sulfuric acid assay results for mannose-coated LNPs using the different solvents in the coupling of Man and LNP-COOH.

The mannose loading on LNP(Gd)-COOH may be increased by changing the reaction conditions for the coupling between LNPs and the mannose derivative. We thus examined the other combinations of coupling agents. For the coupling agents, 9 mM aqueous solutions of *N,N'*-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N,N'*-diisopropylcarbodiimide (DIPC), and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) were examined, while for the promoting agents, 16 mM aqueous solutions of *N*-hydroxysuccinimide (NHS), *N*-hydroxybenzotriazole (HOBt), 2-methyl-6-nitrobenzoic anhydride (MNBA), and

DMAP were used. The combinations of EDC/NHS, DCC/DMAP, HATU/DMAP, DIPC/HOBt, MNBA/DMAP, and PyBOP/DMAP gave the mannose loadings of 35 ± 0.5 , 45 ± 2 , 54 ± 2 , 42 ± 2 , 49 ± 2 , and $31 \pm 1 \mu\text{mol/g}$, respectively. The combination DMAP/HATU gave the highest mannose loading, though the value is different from that described in the previous section for LNP(Gd)-CONH-Man, which is obtained after the optimization of reagent concentration (Figure III-7) and solvent (Figure III-8).

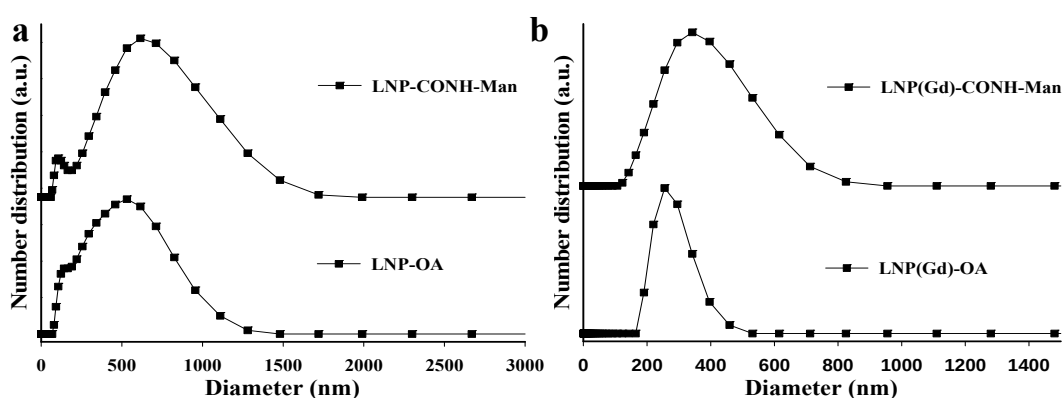


Figure III-9. Diameter distribution of (a) LNP-OA and LNP-CONH-Man and (b) LNP(Gd)-OA and LNP(Gd)-CONH-Man in water, determined by dynamic light scattering (DLS).

For OA-coated LNPs, the hydrodynamic diameters of LNP-OA and LNP-CONH-Man were 775 and 783 nm (Figure III-9a), resulting in severe aggregation. On the other hand, EDHs of LNP(Gd)-OA and LNP(Gd)-CONH-Man were 283 and 385 nm, suggesting that the nanorods are resistant to aggregation (Figure III-9b). In the series of OA-coated LNPs, the addition of mannose did not affect the aggregation tendency so much as that for PEI-coated LNPs.

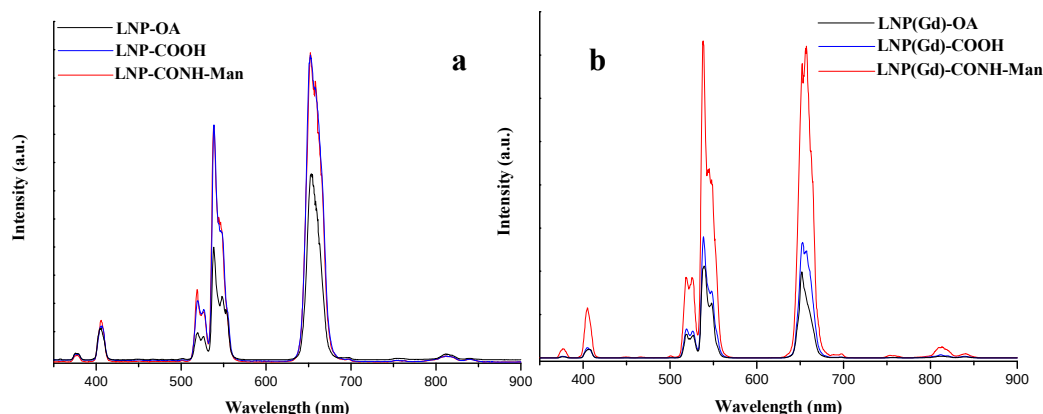


Figure III-10. The upconversion emission spectra of UCLNPs in water before and after mannose unit attachment: (a) LNP-OA series (0.4 mg/mL) and (b) LNP(Gd)-OA series (0.4 mg/mL). The emission spectra were measured with the laser excitaiton power of 400 mW (980 nm) and emission bandwidth of 3 nm.^[13]

The effect of sugar attachment was totally reversed for the series of OA-coated LNPs (Figure III-10a, b), resulting in the increase of emission intensity. The enhancement was particularly drastic for the LNP(Gd)-OA series and reaches almost 3.4-fold for three wavelengths. The luminescence enhancing effect of the sugar-addition is probably not due to the shielding from water, since the solvent quenching is small for OA-coated LNPs as pointed out at the previous section. The enhancement might be caused by microaggregation of LNP(Gd)-CONH-Man.

III-3 Lectin assay of sugar-attached UCLNPs

Considering the aggregation resistance, the large mannose loadings, the absence of size reduction during sugar addition, and the increase of luminescent intensity after mannose attachment, LNP(Gd)-CONH-Man is considered, in view of the structure, the best mannose-modified LNP for biological use among tested.

We tested the interaction of monosaccharides on the surface of LNPs and lectins using lectin aggregation assay. As most lectins exist as a dimer or tetramer, the addition of a multivalent ligand to a lectin solution potentially promotes aggregation formation by making a multiple network

binding of the lectin and ligand.^[9] Lectin aggregation assays are based on the observation of aggregation by a naked eye or turbidity measurement. The turbidity measurement is inappropriate, since it is time-consuming and insensitive. In this regard, using LNP in lectin aggregation assay, the aggregation of a lectin and LNP are monitored by the NIR laser-induced emission of LNP. LNP aggregation will allow a stronger emission than the dispersed one, as opposed to the most fluorophores that give rise to concentration quenching.^[10,11] The incident light within the NIR-window wavelength can be applied for the mixture including autofluorescent biomaterials. The large anti-Stokes shift will facilitate instrumentation, where the aggregation image can be photographed by a digital camera with an NIR cut off filter. The non-photobleaching property will enable a recycled use of LNPs. If we make use of the long life time of LNP emissions,^[12] we will be able to completely cut off the incident light and autofluorescence, allowing a super-sensitive assay.

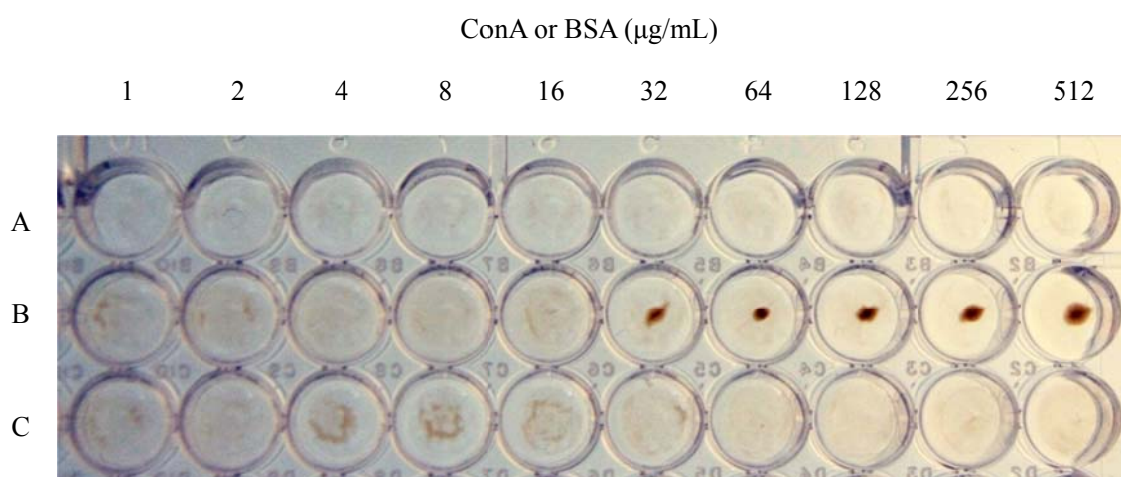


Figure III-11. The aggregation assay of ConA with LNPs: BSA was used as a negative control. Protein concentrations were varied between 1 and 512 $\mu\text{g/mL}$. Lane A: ConA+LNP(Gd)-COOH; Lane B: ConA+LNP(Gd)-CONH-Man; Lane C: BSA+LNP(Gd)-CONH-Man.^[13]

First, we examined a visual inspection of aggregation using LNP(Gd)-CONH-Man and a mannose-binding lectin, concanavalin A (ConA), as shown in Figure III-11. Combinations of ConA+LNP(Gd)-COOH (Lane A), ConA+LNP(Gd)-CONH-Man (Lane B), and bovine serum albumin (BSA)+LNP(Gd)-CONH-Man (Lane C), were examined with protein concentration

varied. The photograph of the microplate was taken from the bottom 1 h after mixing the protein and LNP. Aggregations were clearly visible in five Lane B wells with the ConA concentrations of 32 $\mu\text{g/mL}$ and above. It should be noted here that the photographing of the image in Figure III-11 was not easy, having required a subtle adjustment of lighting, shot angle, and exposure time. For Lanes A and C, no obvious aggregations were observed, indicating that the aggregation in Lane B was caused by a specific binding between Man and ConA. We also tested the aggregated and non-aggregated wells for upconversion emission (Figure III-12). The difference of emission intensities between two irradiated wells is obvious in the photographs, while being more distinct in a naked eye, demonstrating that this procedure is more efficient. The strong emission from the aggregation is an advantage of using LNPs, since most fluorophores suffer from the concentration quenching when they are aggregated.

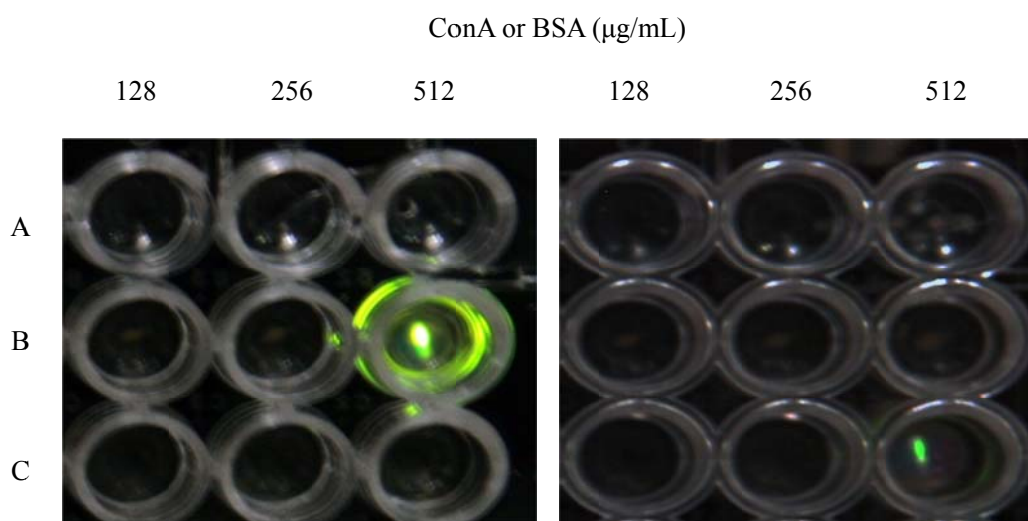


Figure III-12. Upconversion emission of the aggregated (left) and non-aggregated (right) wells in ConA aggregation assay, irradiated by a 980 nm diode laser from the bottom.^[13]

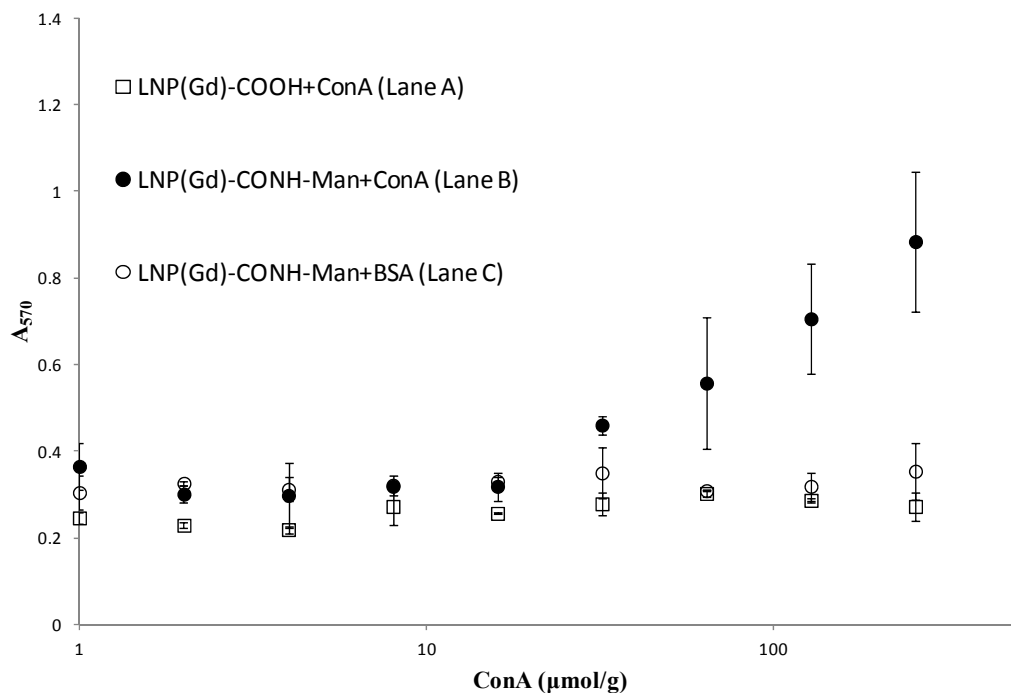


Figure III-13. The ConA aggregation measured by turbidity (A_{570}) with a microplate reader. Protein concentrations: 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 $\mu\text{g/mL}$; LNP(Gd)-CONH-Man concentration: 1 mg/mL .^[13]

We measured the ConA aggregation by turbidity with a microplate reader (Figure III-13). The absorbance at 570 nm (A_{570}) increased obviously with the increase of ConA concentration from 32 $\mu\text{g/mL}$ in Lane B, while those in Lanes A and C stayed the same. The absorptions A_{570} for the ConA concentrations of 64 $\mu\text{g/mL}$ and below are small and indistinguishable, even though the aggregations were visually distinctive. This insensitivity of the turbidity measurement poses an issue for its application.

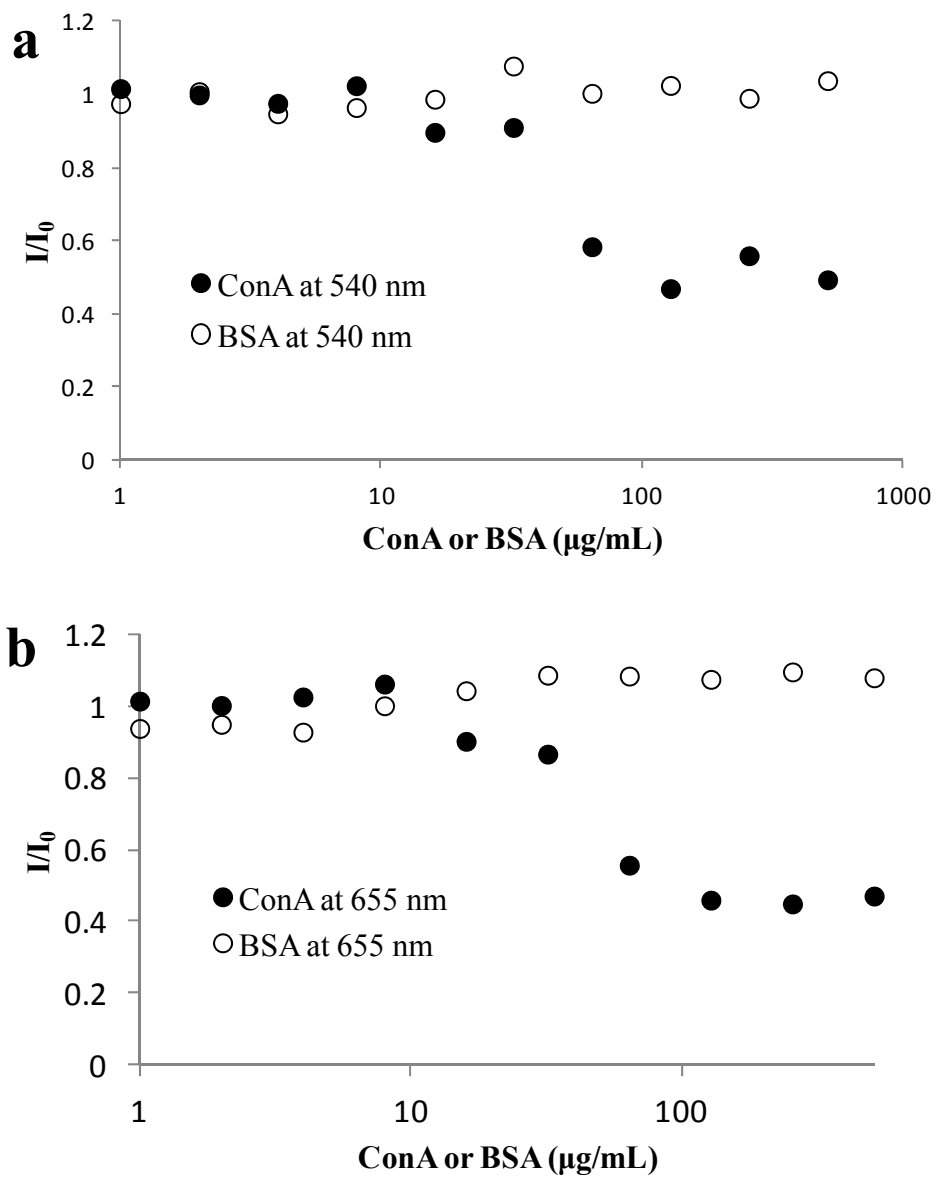


Figure III-14. Upconversion emission intensity of LNP(Gd)-CONH-Man at 540 nm (a) and 655 nm (b) with a supernatant-focus of NIR laser. I_0 is the emission intensity of the blank. Protein concentrations: 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 $\mu\text{g/mL}$; LNP(Gd)-CONH-Man concentration: 1 mg/mL .^[13]

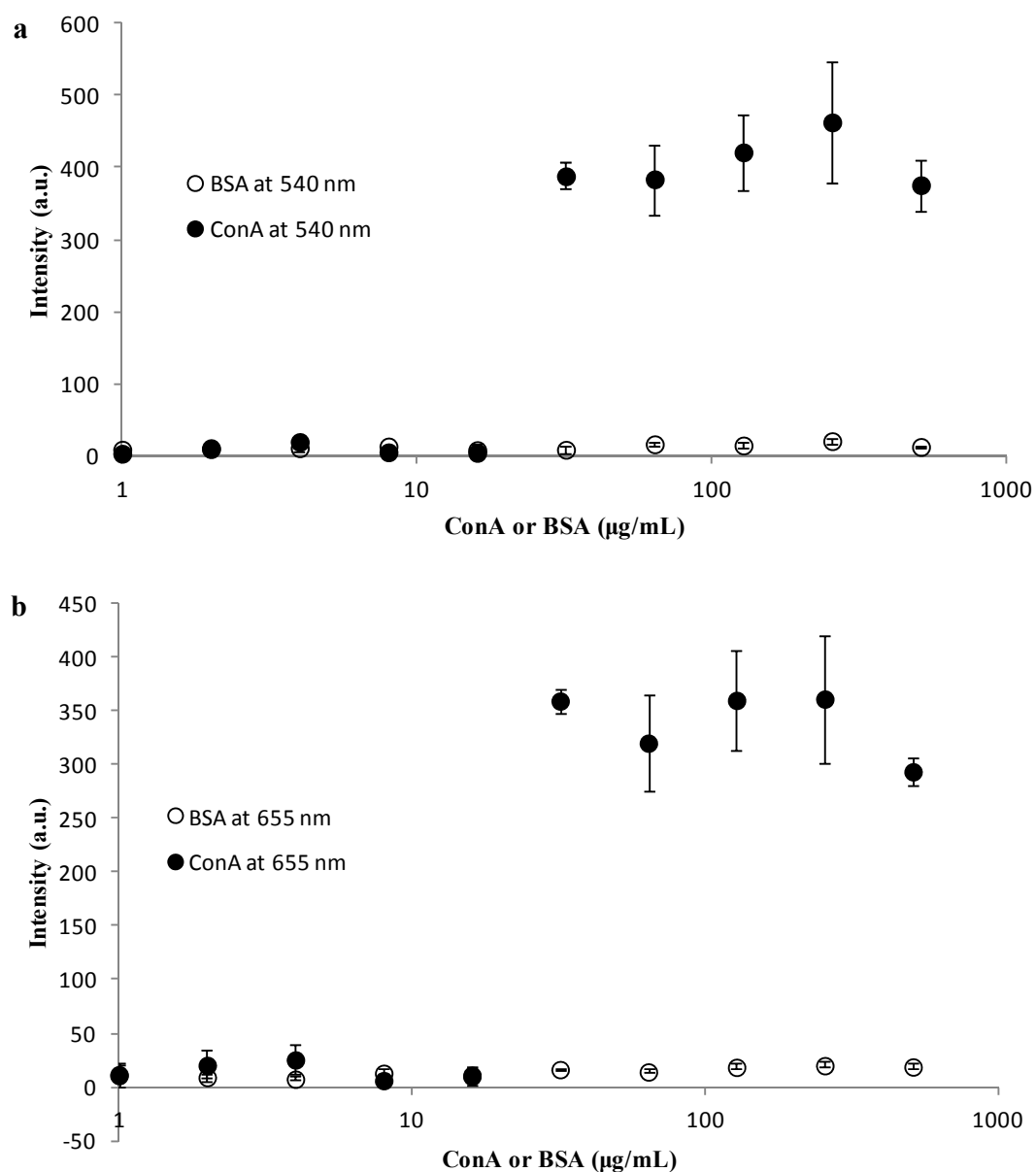
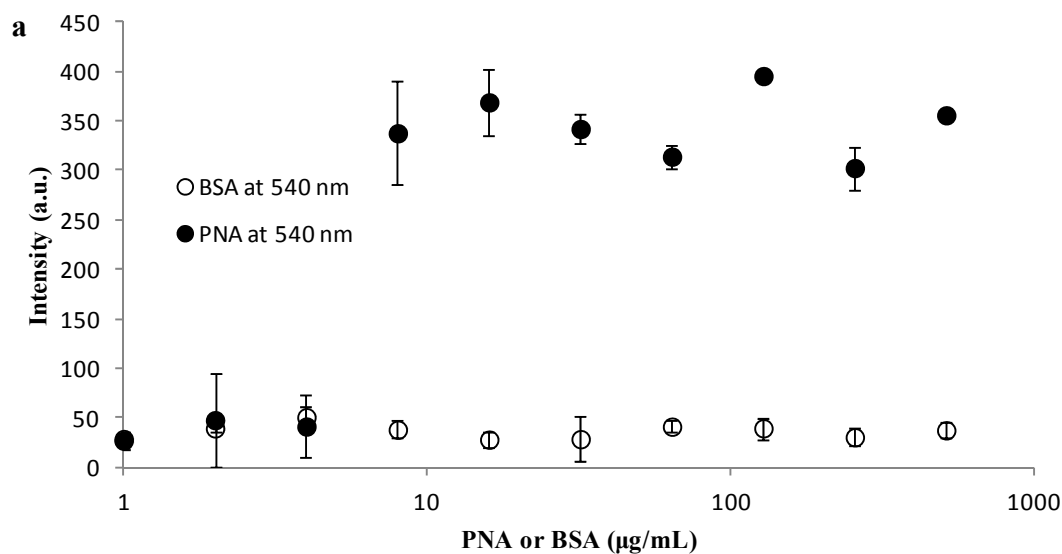


Figure III-15. Upconversion emission intensity of LNP(Gd)-CONH-Man at 540 (a) and 655 (b) nm with a aggregation-focus by NIR laser. Protein concentrations: 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL; LNP(Gd)-CONH-Man concentration: 1 mg/mL. The emission intensities were measured with the laser excitation power of 150 mW (980 nm) and emission bandwidth of 3 nm.^[13]

We next studied the NIR-excited emission spectra for the mixture of LNP(Gd)-CONH-Man and ConA. The emissions at 540 and 655 nm were measured with the NIR-laser focus at the supernate fluid in a glass tube or the aggregation in a polypropylene microtube. The supernatant focus gave reduction of the emission intensity at 540 (Figure III-14a) and 655 nm (Figure III-14b) with the

increase of [ConA], while aggregation focus did increase the intensity at 540 and 655 nm (Figures II-18). In the case of the supernatant focus, the lower limit of [ConA] for the aggregation recognition was 64 $\mu\text{g/mL}$, having improved from the turbidity assay. However, the I/I_0 variation was not large and sensitive enough. This insensitivity of the supernate focus method was expected from the fact that the incident light is focusing on a darker site of the analyte. The aggregation-focused assay clearly distinguished the aggregated and non-aggregated samples, the difference of emission intensities being more than six times, even though an opaque polypropylene microtube was used as an assay vessel. Similar results were obtained with LNP(Gd)-CONH-Gal and peanut agglutinin(PNA), a galactose-recognizing lectin (Figure III-16), and thus the aggregation with the PNA concentrations of 8 $\mu\text{g/mL}$ and above was clearly distinguished from the non-aggregated samples. These results are quite encouraging for recognizing the interaction between monosaccharides on the surface of sugar-attached LNPs and lectins.



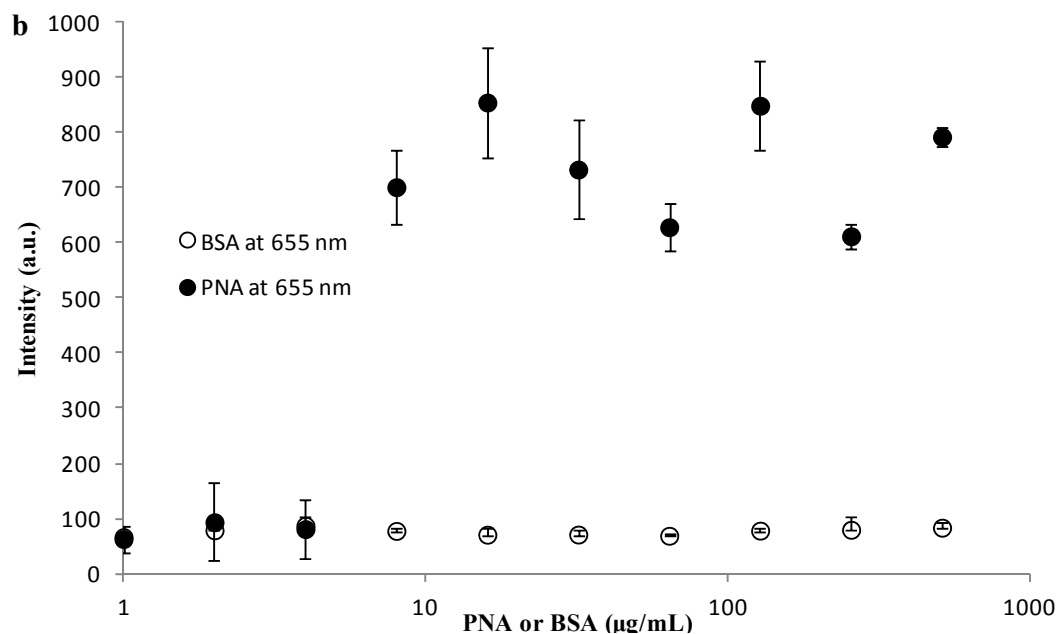


Figure III-16. Upconversion emission intensity of LNP(Gd)-CONH-Gal at 540 (a) and 655 (b) nm with a aggregation-focus by NIR laser. Protein concentrations: 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 $\mu\text{g/mL}$; LNP(Gd)-CONH-Gal concentration: 1 mg/mL. The emission intensities weremeasured with the laser excitation power of150 mA (980 nm)andemission bandwidth of 3 nm.^[13]

III-4 Macrophage assay of sugar-attached UCLNPs

In order to target a tumor tissue, one should know the best monosaccharide ligand bound by the tumor-associated lectins. In this context, the sugar-LNP conjugates may also be used for the rapid identification of the sugar bound by the target tissue with a method similar to the lectin aggregation assay described in the previous section. In a related study, we had demonstrated that the mouse macrophage cell line RAW 264.7 was aggregated with a multivalent galactose ligand, suggesting that a galectin-like lectin was exposed on the cell (unpublished result). We therefore tested LNP-GA-Gal and LNP(Gd)-CONH-Gal for the macrophage aggregation assay. LNPs were mixed well with RAW 264.7 and the mixtures were transferred to a 96 well microplate. After 24 h, formation of aggregations for the LNP-mixed cells as compared with the negative controls was barely observed by visual inspection with a microscope ($\times 40$). However, after transferring the

mixtures to microtubes, the precipitates of Gal-attached LNPs showed a luminescence distinct from the negative controls on irradiation with an NIR laser diode (insets in Figure III-17): the sugar-LNP mixed with RAW264.7 showed a strong yellowish emission, while a negative control with a bare LNP showed a weak reddish emission. The difference of luminescence spectra between these precipitates was more obvious than they look in the photographs (Figure III-18): the emission retardation for the negative control in the green region (540 nm) was more drastic than that for the red light (655 nm) in consistent with the yellow-to-red color change (Figure III-17). It is suggested that the precipitates of the mixture of LNP-GA-Gal and cells incorporate a larger number of LNPs than those of the negative controls owing to cell-to-cell bridging by the multivalent galactose on LNPs. Although we tested the strongest emitter, LNP(Gd)-CONH-Gal, as a macrophage assay tool, the emission from the precipitates of RAW264.7 showed no difference from the negative controls on irradiation with an NIR laser (Figure III-19). This may be due to the rod-like shape of OA coated Gd doped LNPs, which may restrict the orientation of galactose and limit the interaction with the cell surface lectins. We thus demonstrated that sugar-attached LNPs could be exploited in a facile and rapid assay for the sugar specificities of cell surface lectins, which will serve useful information to the design of tumor-targeting drug delivery systems and used in PDT experiment *in vivo*.

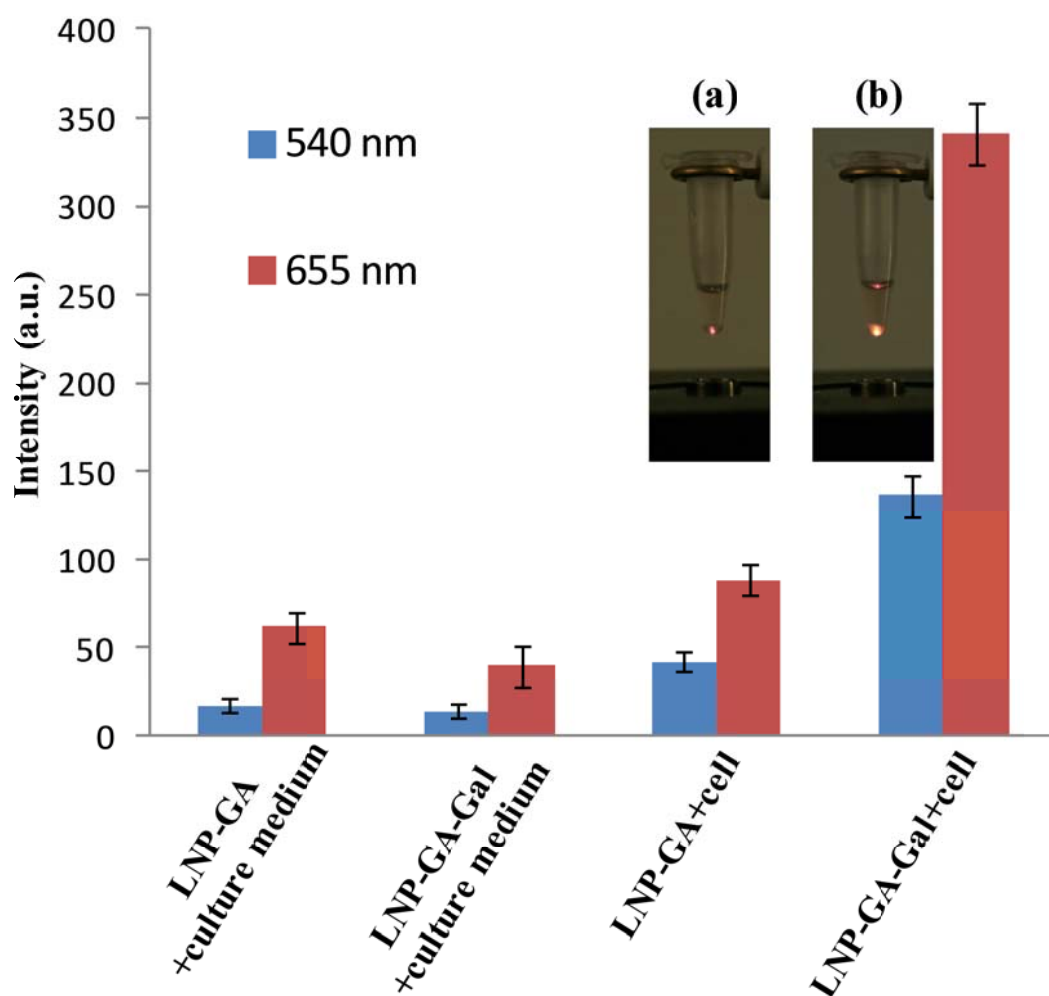


Figure III-17. Upconversion emission intensity for the aggregation of macrophage RAW 264.7 with LNP-GA or LNP-GA-Gal (2 mg/mL) and culture medium with LNP-GA or LNP-GA-Gal (2 mg/mL) at 540 and 655 nm. The emission intensities were measured with the laser excitation power of 400 mW (980 nm) and emission bandwidth of 3 nm. The insets show the emission from LNP-GA (a) and LNP-GA-Gal (b) in the aggregation excited with 1 W laser diode (975 nm).^[13]

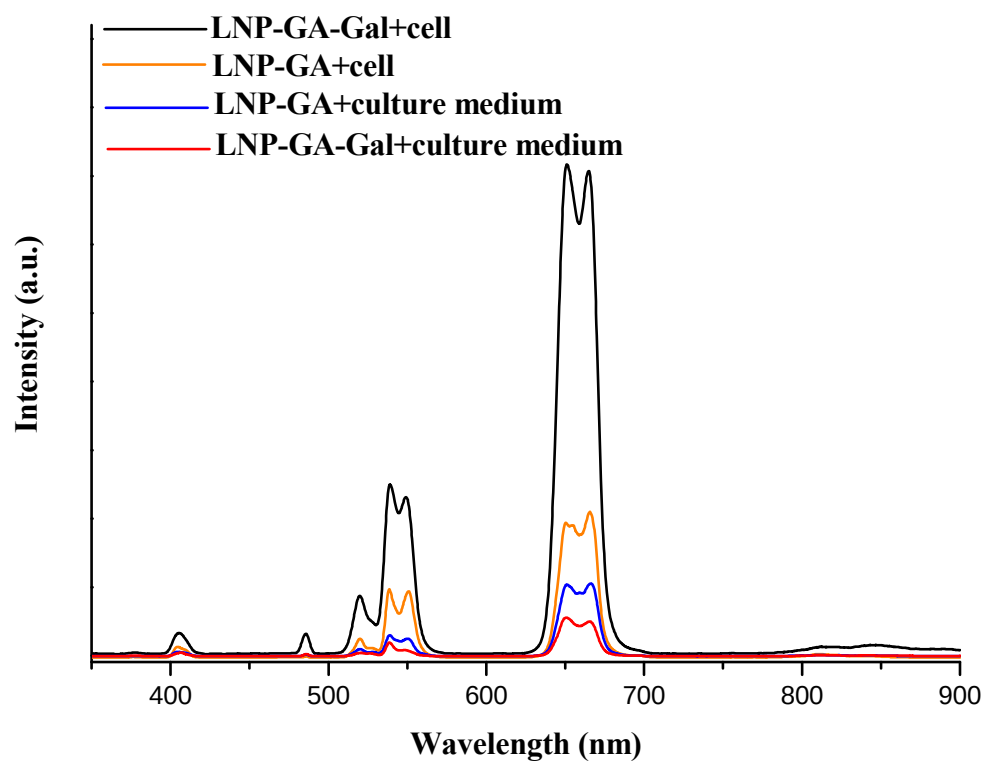


Figure III-18. The upconversion emission spectra for the aggregations of LNP-GA-Gal+cell, LNP-GA+cell, LNP-GA+culture medium and LNP-GA-Gal+culture medium in the macrophage assay. The emission spectra were measured with the laser excitation power of 400 mW (980 nm) and emission bandwidth of 3 nm.^[13]

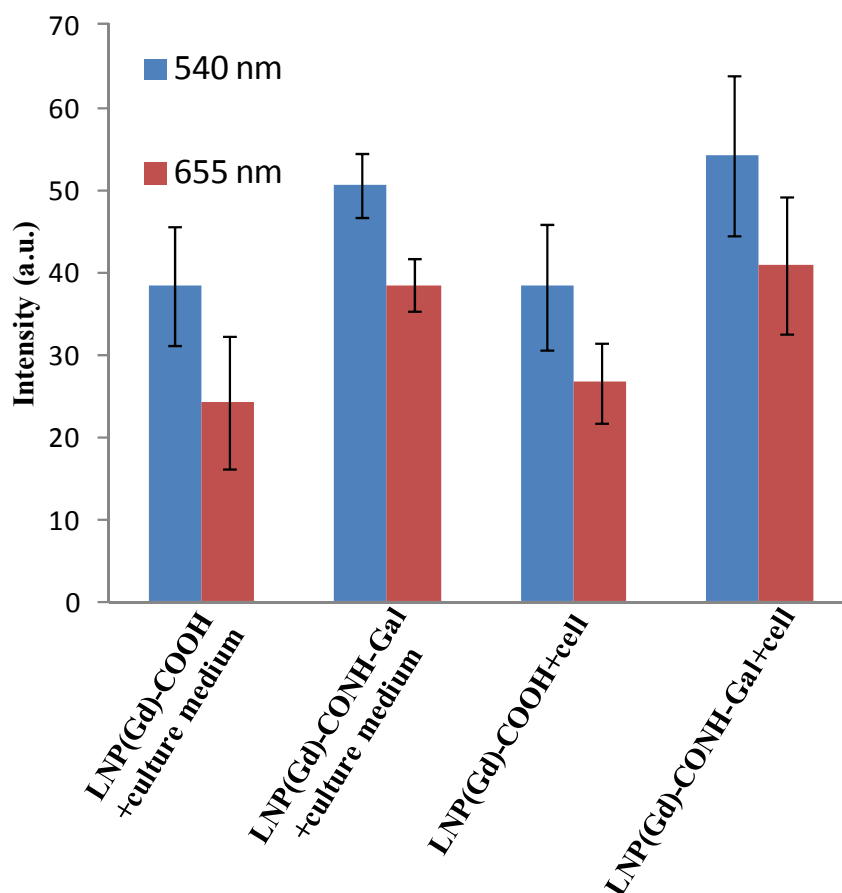


Figure III-19. Upconversion emission intensity for the aggregation of macrophage RAW 264.7 with LNP(Gd)-COOH or LNP(Gd)-CONH-Gal (2 mg/mL) and culture medium with LNP(Gd)-COOH or LNP(Gd)-CONH-Gal (2 mg/mL) at 540 and 655 nm. The emission intensities were measured with the laser excitation power of 100 mW (980 nm) and emission bandwidth of 3 nm.^[13]

III-5 Summary

In this chapter, we synthesized PEI- and OA-coated upconversion LNPs, NaYF₄:Yb/Er(/Gd), conjugated with a mannose or galactose unit through covalent bonds. For PEI-coated LNPs, the amino group was treated with glutaric anhydride (GA) and the resulting carboxylic ends were reacted with 2-aminoethyl mannoside (Man) to give LNP-GA-Man and LNP(Gd)-GA-Man. In the case of OA-coated LNPs, the OA alkene was oxidized to -COOH, which was then coupled with

Man to give LNP-CONH-Man and LNP(Gd)-CONH-Man. TEM and XRD measurements showed that the previously unreported LNP(Gd)-NH₂ had a nanocube morphology composed of cubic phase crystals. LNP(Gd)-NH₂ demonstrated severe erosion after the sugar modification, giving a smaller size relative to LNP(Gd)-NH₂ and a low sugar loading ($4.0 \pm 1.2 \mu\text{mol/g}$). The other LNPs underwent little damage during sugar modification and the sugar loadings were 47 ± 3 , 56 ± 5 , and $93 \pm 0.5 \mu\text{mol/g}$ for LNP-GA-Man, LNP-CONH-Man, and LNP(Gd)-CONH-Man, respectively. The aggregation properties of LNP solutions were assessed by DLS, demonstrating that LNPs with cubic phase resulted in severe aggregations. Only the nanorodes, LNP(Gd)-OA and LNP(Gd)-CONH-Man, had the effective hydrodynamic diameters comparable to the TEM-shot particle sizes, indicating less aggregation. When mannose was attached to OA-coated LNPs, about 3-fold intensity increases were observed, whereas mannose attachments for LNP(Gd)-NH₂ and LNP-NH₂ ended in signal reductions by almost 50%. Overall, LNP(Gd)-CONH-Man was found to be the best candidate among synthesized LNPs for its high mannose loading, low aggregation tendency, and high emission intensity. In addition, a lectin aggregation assay with LNP(Gd)-CONH-Man showed that the aggregation due to the binding between LNP and ConA was unambiguously distinguished from the non-aggregated samples by the strong emission from the aggregated LNP. The distinction was more than six-fold even for the sample that gives only an absorbance difference within the error margin in a turbidity measurement. We tested the sugar-attached LNPs for the assay that evaluates the sugar specificity of cell surface lectins. As a result, the galactose specificity of macrophage cell line RAW 264.7 was easily identified by evaluating the NIR-excited luminescence from the precipitates of the cells incorporating LNP-GA-Gal: a relatively strong and yellowish emission was observed from LNP-GA-Gal-incorporated precipitates, while a weaker and reddish emission was seen for all the negative controls. Overall, sugar-attached LNPs were the best candidates used in PDT experiment.

Reference

- [1] L. Xiong, Z. Chen, Q. Tian, T. Cao, C. Xu, F. Li, High contrast upconversion luminescence targeted imaging in vivo using peptide-Labeled nanophosphors, *Anal. Chem.* **2009**, *81*,

8687-8694.DOI: 10.1021/ac901960d.

[2] S. A. Hilderbrand, F. Shao, C. Salthouse, U. Mahmood, R. Weissleder, Upconverting luminescent nanomaterials: application to in vivo bioimaging, *Chem. Commun.* **2009**, 4188-4190. DOI: 10.1039/b905927j.

[3] L. Cheng, K. Yang, Y. Li, J. Chen, C. Wang, M. Shao, S.-T. Lee, Z. Liu, Facile preparation of multifunctional upconversion nanoprobe for multimodal imaging and dual-targeted photothermal therapy, *Angew. Chem. Int. Ed.* **2011**, 50, 7385-7390. DOI: 10.1002/anie.201101447.

[4] H. Xing, W. Bu, S. Zhang, X. Zheng, M. Li, F. Chen, Q. He, L. Zhou, W. Peng, Y. Hua, J. Shi, Multifunctional nanoprobe for upconversion fluorescence, MR and CT trimodal imaging, *Biomaterials* **2012**, 33, 1079-1089. DOI:10.1016/j.biomaterials.2011.10.039.

[5] J. Hampl, M. Hall, N. A. Mufti, Y. M. Yao, D. B. MacQueen, W. H. Wright, D. E. Cooper, Upconverting phosphor reporters in immunochromatographic assays, *Anal. Biochem.* **2001**, 288, 176-187. DOI:10.1006/abio.2000.4902.

[6] H. Guo, R. Hao, H. Qian, S. Sun, D. Sun, H. Yin, Z. Liu, X. Liu, Upconversion nanoparticles modified with aminosilanes as carriers of DNA vaccine for foot-and-mouth disease, *Appl. Microbiol. Biotechnol.* **2012**, 95, 1253-1263. DOI 10.1007/s00253-012-4042-z.

[7] N. Sharon, H. Lis, History of lectins: from hemagglutinins to biological recognition molecules, *Glycobiology* **2004**, 14, 53-62. DOI:10.1093/glycob/cwh122.

[8] Z. Chen, H. Chen, H. Hu, M. Yu, F. Li, Q. Zhang, Z. Zhou, T. Yi, C. Huang, Versatile synthesis strategy for carboxylic acid-functionalized upconverting nanophosphors as biological labels, *J. Am. Chem. Soc.* **2008**, 130, 3023-3029. DOI: 10.1021/ja076151k.

[9] H. Yuasa, T. Haraguchi, T. Itagaki, A facile latex agglutination lectin assay (LALA) for

weakly binding ligands, *J. Carbohydr. Chem.* **2009**, *28*, 78-93. DOI: 10.1080/07328300802696207.

[10] K. D. Chaudhuri, Concentration quenching of fluorescence in solutions, *Z. Phys.* **1959**, *154*, 34-42.

[11] Y. Hong, J. W. Y. Lam, B. Z. Tang, Aggregation-induced emission, *Chem. Soc. Rev.* **2011**, *40*, 5361-5388. DOI: 10.1039/c1cs15113d.

[12] L. D. DeLoach, S. A. Payne, L. L. Chase, L. K. Smith, W. L. Kway, W. F. Krupke, Evaluation of absorption and emission properties of Yb^{3+} doped crystals for laser applications, *IEEE J. Quantum Electron.* **1993**, *29*, 1179-1191.

[13] Y. Liu, T. Kobayashi, M. Iizuka, T. Tanaka, I. Sotokawa, A. Shimoyama, Y. Murayama, E. Otsuji, S. Ogura, H. Yuasa, Sugar-attached upconversion lanthanide nanoparticles: a novel tool for high-throughput lectin assay, *Bioorg. Med. Chem.* **2013**, *21*, 2832-2842. DOI: 10.1016/j.bmc.2013.04.008.

Chapter IV Using synthesized UCLNP in PDT experiments

IV-1 Introduction

With synthesized LNPs in hand, PDT experiments *in vitro* can be carried out using LNPs. In this chapter, boric acid doped LNPs synthesized in Chapter I was used because of its significant luminescent intensity at 410, 540 and 655 nm.

Before using these LNPs into PDT experiments, the cell toxicity of LNPs should be under investigation. The protocol of MTT (MTT: (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was shown in Figure IV-1. MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple color. MKN45 cell, a cancer cell line from stomach cancer of human, was incubated with trypsin at 37 °C, 5% CO₂ for 5 min. The number of living cells was counted by hemocytometer and the solution containing cells was centrifugated. And then the cells were placed into 96 well plates and cultured overnight. The culture medium was replaced with LNPs solution at different concentrations and cultured overnight. MTT solution was added and incubated for 4 h. 10% SDS (sodium dodecyl sulfate) solution was added and incubated overnight. The absorbance at 570-660 nm for each well was measured. The percentage of cell viability for each sample was calculated by comparing the absorbance with 100% viability samples (PBS buffer as positive control) and 0% viability samples (SDS as negative control).

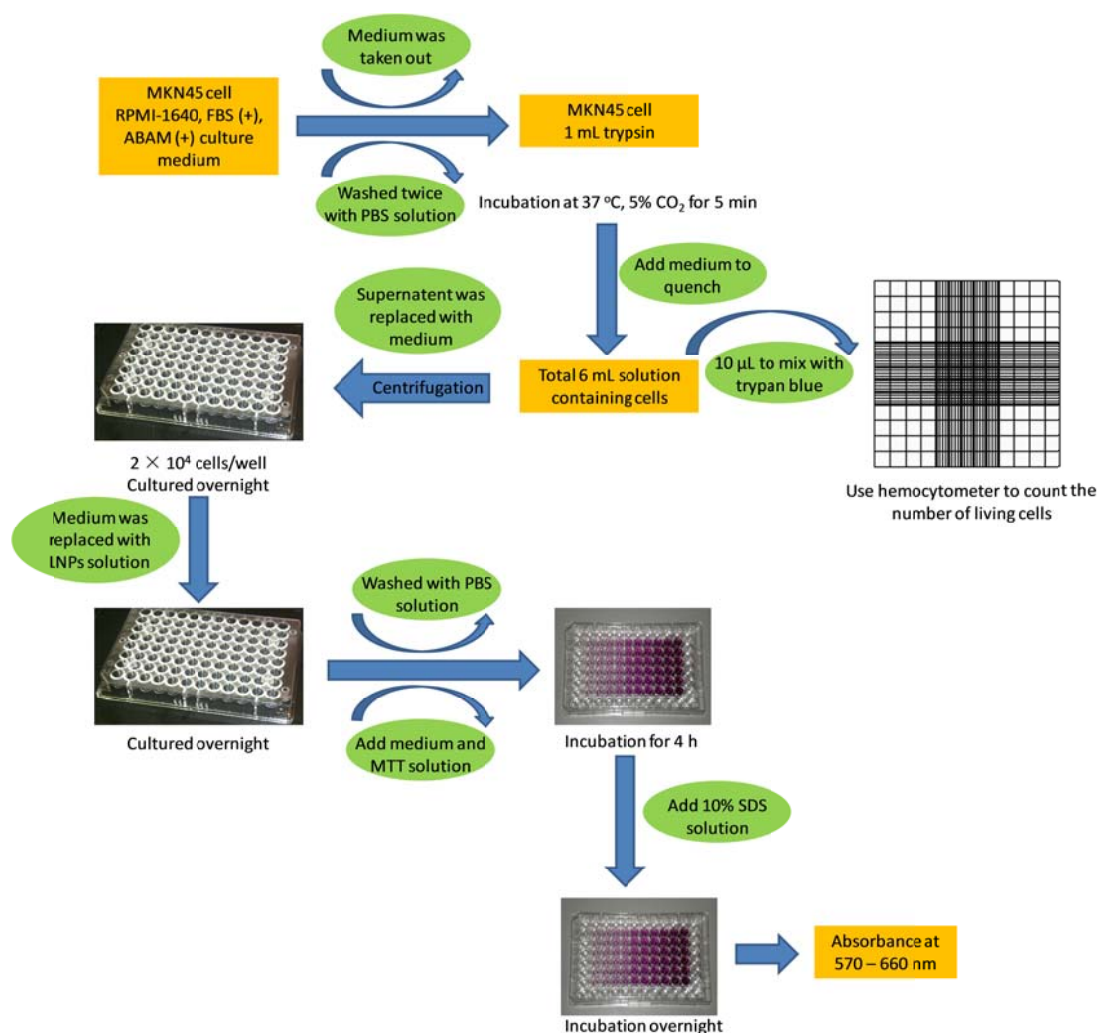


Figure IV-1. The protocol of MTT assay to investigate the cell toxicity of LNPs.

The protocol of using LNPs in PDT experiment *in vitro* was similar to MTT assay as shown in Figure IV-2. MKN45 cell was incubated with trypsin at 37 °C, 5% CO₂ for 5 min. The number of living cells was counted by hemocytometer and the solution containing cells was centrifuged. And then the cells were placed into 96 well plates and cultured overnight. The culture medium was replaced with ALA at different concentrations together with 2 mg/mL tested LNPs and cultured overnight. Then, the plates were irradiated under NIR laser (980 nm, 400 mA) 50 mm above for 90 min. After that, MTT solution was added and incubated for 4 h. 10% SDS (sodium dodecyl sulfate) solution was added and incubated overnight. The absorbance at 570-660 nm for each well was measured.

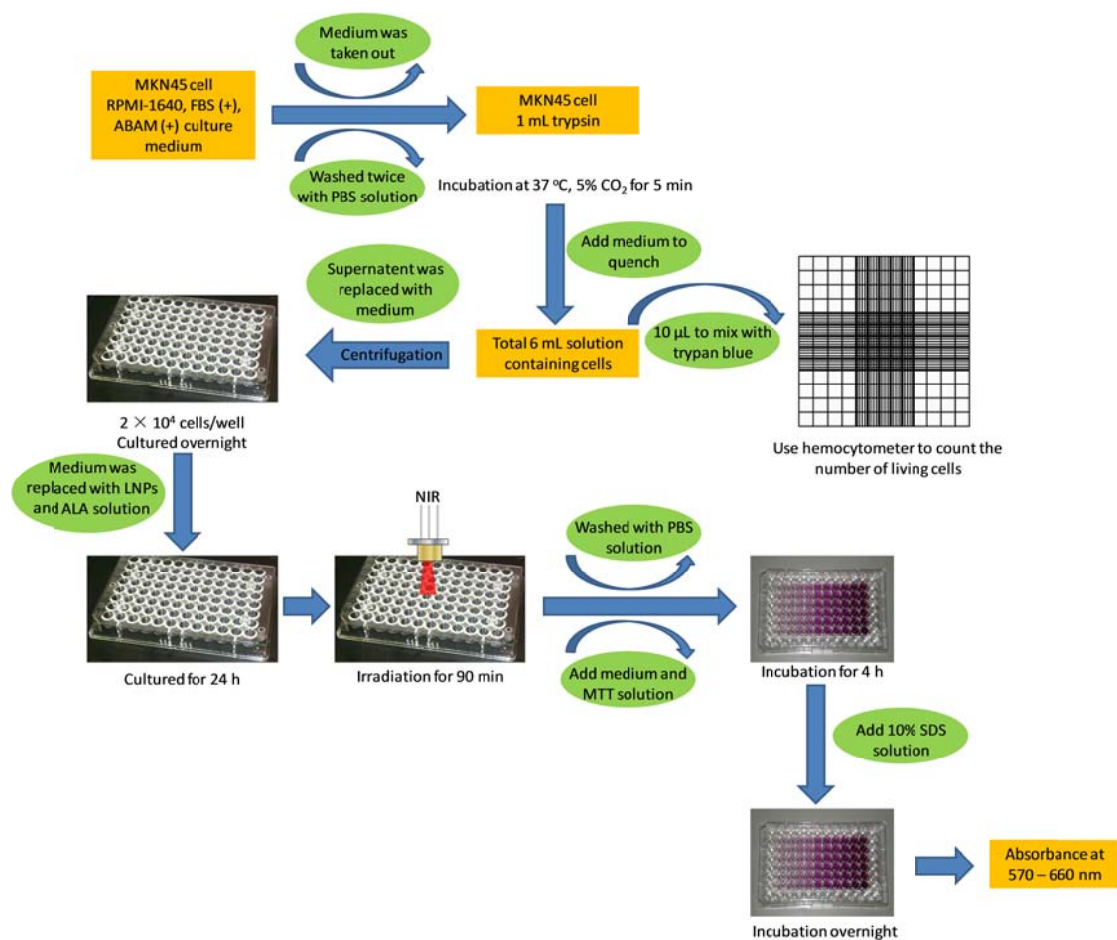


Figure IV-2. The protocol of using synthesized LNPs in PDT experiment.

IV-2 MTT assay of synthesized UCLNP

The MTT assay was followed the protocol shown as Figure IV-1. The result of MTT assay of boric acid doped LNPs was shown in Figure IV-3. In this result, it is clearly that the cell viability was remained over 90% with the increase of LNPs concentration from 0 to 2.2 mg/mL. At 2.5 mg/mL, the cell viability was under 80% and even lower than 40% at LNPs concentration of 4 mg/mL.

According to this result, we used 2 mg/mL boric acid doped LNPs in PDT experiment.

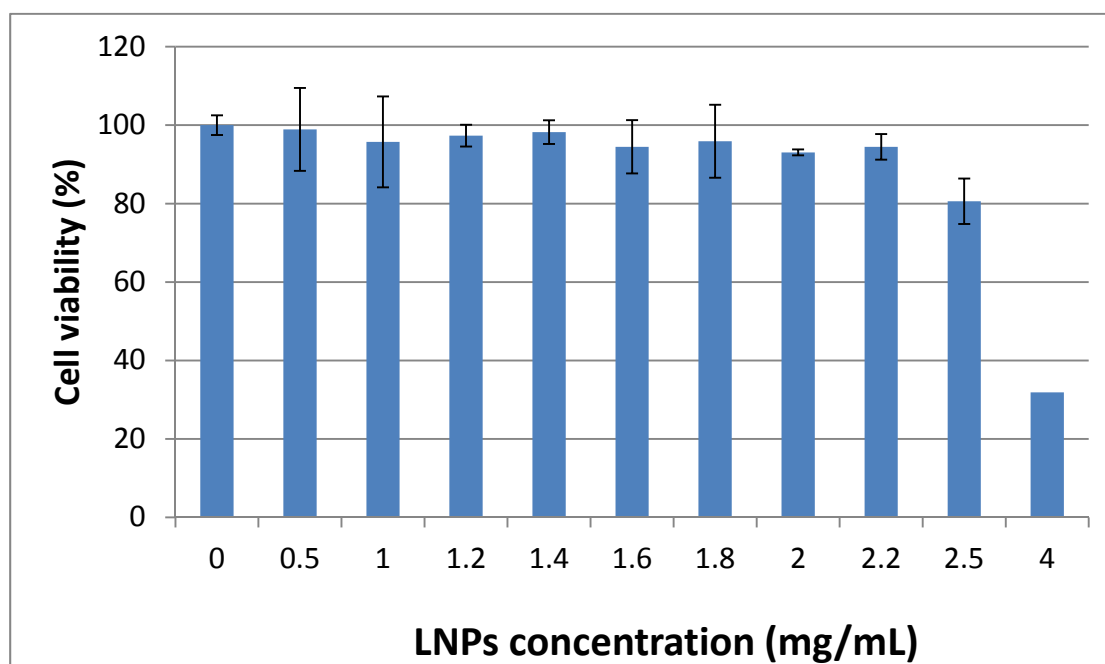


Figure IV-3. The cell toxicity of boric acid doped LNPs using MTT assay.

IV-3 PDT experiments of synthesized UCLNP

The PDT experiment of boric acid doped LNPs was followed the protocol shown as Figure IV-2. The result was shown in Figure IV-4.

As shown in Figure IV-4, the cell viability was decreased obviously with the concentration of ALA over 1000 μM . When the concentration of ALA reached 2000 μM , the cell viability was around 60%, which means around 40% of cells were killed by singlet oxygen reacted from PPIX excited by the emission of boric acid doped LNPs.

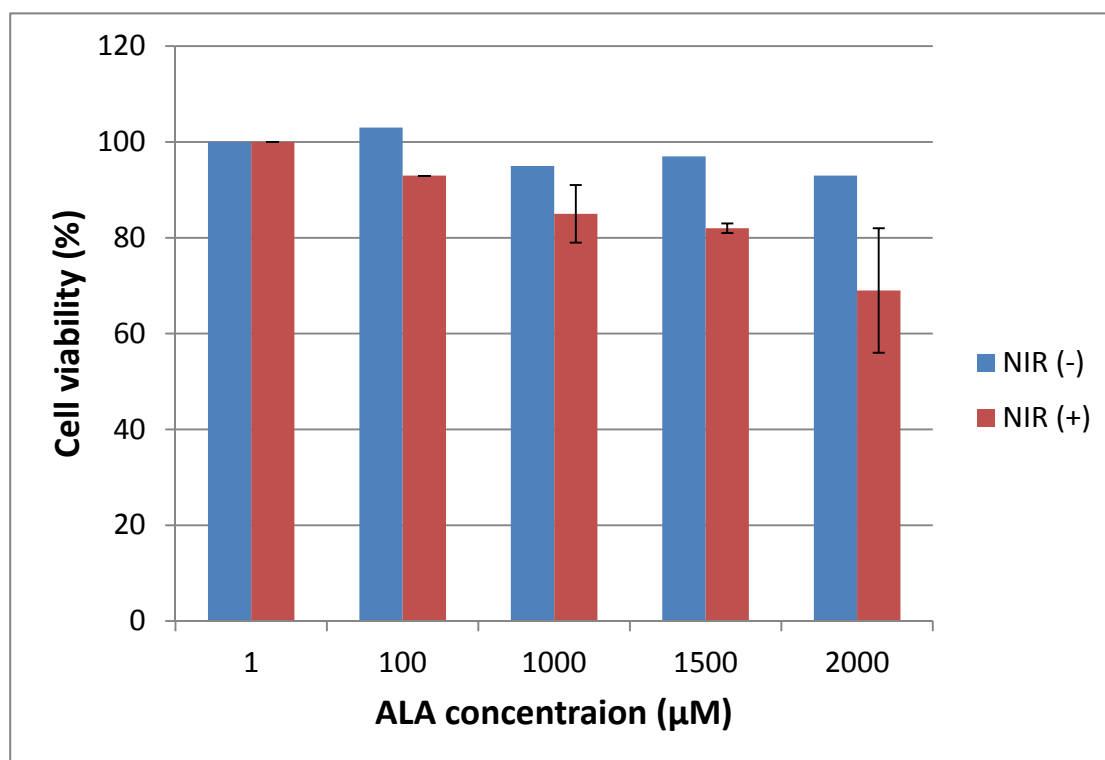


Figure IV-4. PDT experiment of boric acid doped LNPs.

IV-4 Summary

In this chapter, boric acid doped NaYF₄:Yb/Er (18/2 mol%) upconversion nanoparticles were applied in PDT experiment. The MTT assay showed that the cell viability remained over 90% until the concentration of LNPs reached 2.2 mg/mL. 40% of cells were killed with 2 mg/mL LNPs and 2000 μM ALA used in PDT experiment.

Chapter V Conclusion

Photodynamic therapy (PDT) is a kind of photo medical method using nontoxic light-sensitive compounds which become toxic to targeted cells when they are exposed selectively to light. Thus photodynamic therapy is an easy, not expensive method to cure cancer. However ordinary PDT is only efficient for surface cancer because the light source of photosensitizer is suffered from low penetration depth. In order to overcome this disadvantage, upconversion lanthanide nanoparticles are used in PDT. Upconversion nanoparticles can convert near-infrared radiation (NIR) into visible light which can be used to excite photosensitizer. Comparing to organic fluorophores or semi-conducting nanocrystals (as quantum dots), upconversion lanthanide nanoparticles are more proper to be used in PDT because LNPs have large anti-Stokes shift and sharp peaks, the ability of absence of autofluorescence from biological samples, high penetration depth, and non-photobleaching and non-photoblinking properties.

In the first chapter, different kinds of upconversion nanoparticles were synthesized. Among the tested samples, oleic acid coated NaYF₄:Yb/Er/Gd (18/2/30 mol%) nanoparticles and boric acid doped NaYF₄:Yb/Er (18/2 mol%) nanoparticles were the best choices used in the application of PDT because both of them showed high luminescent intensity at 540 and 655 nm.

In the second chapter, sugar-attached LNPs were synthesized and investigated. The linkers and coupling reagents used to synthesize mannose or galactose attached LNPs through PEI or OA coats were under investigation. LNP(Gd)-CONH-Man and LNP(Gd)-CONH-Gal were used in the ConA and PNA aggregation assays, respectively. Macrophage aggregation assay was carried out successfully using LNP-GA-Gal upconversion nanoparticles.

In the third chapter, boric acid doped NaYF₄:Yb/Er (18/2 mol%) nanoparticles were used in the MTT assay and PDT experiment *in vitro*. The cell viability of MTT assay remained over 90% until the concentration of LNPs reached 2.2 mg/mL. Over 40% of cells were killed in PDT experiment with 2 mg/mL LNPs and 2000 μ M ALA.

In conclusion, lanthanide doped upconversion nanoparticles can be used in photodynamic therapy to overcome the shortcoming of low penetration depth by using NIR (980 nm) light source. Further studies are required to synthesize more efficient upconversion nanoparticles and use them in PDT experiment *in vivo*.

Experimental section

Materials

All starting materials were obtained from commercial suppliers and used as received. Rare earth chlorides $\text{YCl}_3 \cdot 6\text{H}_2\text{O}$ (99.99 %), $\text{YbCl}_3 \cdot 6\text{H}_2\text{O}$ (99.99 %), $\text{ErCl}_3 \cdot 6\text{H}_2\text{O}$ (99.99 %), $\text{GdCl}_3 \cdot x\text{H}_2\text{O}$ (99.99 %), polyethylenimine (PEI, branched, 25 kDa) and all the coupling reagents were purchased from Sigma-Aldrich Co. Oleic acid (OA) was purchased from Kanto Chemical Co. Inc. Potassium permanganate and sodium periodate were purchased from Kokusan Chemical Co., Ltd. NaOH, NH_4F , hydrochloric acid, ethanol, cyclohexane, *t*-butyl alcohol, ethylene glycol, DMF, phenol, sulfuric acid, and K_2CO_3 were purchased from Wako Pure Chemical Industries Ltd. Concanavalin A (ConA) from *Canavalia ensiformis* (Jack bean) Type IV lyophilized powder and bovine serum albumin (BSA) lyophilized powder were purchased from Sigma-Aldrich Co. PNA, lectin from *Arachis hypogaea* (peanut), lyophilized powder were purchased from J-OIL mills, Inc. All other chemical reagents with analytical grade were used directly without further purification. Deionized (DI) water was used throughout.

Characterization

Upconversion nanoparticles were synthesized in a high pressure micro-reactor, MMJ 100 (OM Labotech Ltd.). The size and morphology of upconversion nanoparticles were determined with a JEM-2010F field emission transmission electron microscopy (FE-TEM) at 200 kV. Powder X-ray diffraction (XRD) measurements were performed on an X-ray diffractometer for pole point figure measurement, X'Pert-MPD-OEC, in the 2θ range between 15° and 100° . The upconversion luminescence emission spectra were recorded on a Shimadzu RF-5300PC spectrofluorophotometer equipped with an external laser diode excitation source (300 mW, 980 nm, THORLABS, Inc.). UV-visible absorbance was recorded with a UVmini-1240 photospectrometer (Shimadzu Co.). IR spectra were recorded with a Shimadzu FTIR-8400S Fourier transform IR spectroscopy. Dynamic light scattering (DLS) measurements were carried

out on a Zetasizer nano series from Malvern Instruments Ltd. TEM and XRD data were obtained from Center for Advanced Materials Analysis of National University Corporation Tokyo Institute of Technology. All the photoluminescence studies were carried out at room temperature. Microplate aggregation assay was conducted on a Bio-Rad Model 550 microplate reader equipped with a 570 nm filter to evaluate the turbidity of the assay samples. Culture medium of MTT assay and PDT experiment was RPMI 1640 with L-glutamine purchased from Nacalai Tesque. It contains: Calcium Nitrate • 4H₂O 100.00, Magnesium Sulfate 48.84, Potassium Chloride 400.00, Sodium Chloride 6000.00, Sodium Hydrogen Carbonate 2000.00, *di*-Sodium Hydrngenphosphate 800.00, *L*-Arginine 200.00, *L*-Aspartic Acid 20.00, *L*-Asparagine 50.00, *L*-Cystine • 2HCl 65.20, *L*-Glutamic Acid 20.00, *L*-Glutamine 300.00, Glycine 10.00, *L*-Histidine 15.00, Hydroxy-*L*-proline 20.00, *L*-Isoleucine 50.00, *L*-Leucine 50.00, *L*-Lysin•HCl 40.00, *L*-Methionine 15.00, *L*-Phenylalanine 15.00, *L*-Proline 20.00, *L*-Serine 30.00, *L*-Threonine 20.00, *L*-Tryptophan 5.00, *L*-Tyrosine • 2Na • 2H₂O 28.83, *L*-Valine 20.00, *p*-Aminobenzoic Acid 1.00, *D*-Biotin 0.20, Choline Chloride 3.00, Folic Acid 1.00, *myo*-Inositol 35.00, Nicotinamide 1.00, *D*-Pantothenic Acid Calcium Salt 0.25, Pyridoxine • HCl 1.00, Vitamine B₁ • HCl 1.00, Vitamin B₂ 0.20, Vitamin B₁₂ 0.005, D-Glucose 2000.00, Glutathione 1.00, Phenol Red Sodium 5.30, 10% FBS (fetal bovine serum purchased from Nacalai Tesque) and 1% ABAM (antibiotic-antimycotic mixed stock solution (100 x) (stabilized) purchased from Nacalai Tesque).

Chapter II

Synthesis of OA-coated NaYF₄:Yb/Er/Gd (18/2/x mol% x = 0, 30, 45, 60 and 75)

The synthetic procedure followed that of F. Wang, et al.: in a typical procedure, a solution of 1.2 g NaOH in water (6 mL) was mixed with 20 mL of ethanol and 20 mL of oleic acid under stirring. To the resulting mixture were added RECl₃ (0.2 M, RE = 1.3 mmol Y, 0.29 mmol Yb, 0.032 mmol Er, or 0.80 mmol Y, 0.29 mmol Yb, 0.032 mmol Er, 0.48 mmol Gd, or 0.56 mmol Y, 0.29 mmol Yb, 0.032 mmol Er, 0.72 mmol Gd, or 0.32 mmol Y, 0.29 mmol Yb, 0.032 mmol Er, 0.96 mmol Gd, or 0.08 mmol Y, 0.29 mmol Yb, 0.032 mmol Er, 1.2 mmol Gd) in water (8 mL) and 2 M NH₄F (4 mL). Then the solution was transferred into a 100 mL of crystal glass reaction vessel, set for the

autoclave, and heated at 200 °C for 2 h (1 h for 0% Gd) with stirring in the sealed stainless cylinder. The obtained nanoparticles were collected by centrifugation, washed with ethanol and water several times, and dried in vacuo to give OA-coated NaYF₄:Yb/Er/Gd (18/2/x mol% x = 0, 30, 45, 60 and 75).

Synthesis of boric acid doped NaYF₄:Yb/Er (18/2 mol%)

In a typical synthesis, 2 mL of 0.5 mol/L RECl₃ (RE = Y/Yb/Er 80/18/2 mol%) aqueous solution and 2 mmol of boric acid were dispersed into 40 mL of deionized water and magnetically stirred for 1 h, forming a chelated RE-B(OH)₃ complex. Then 16 mL of 0.5 mol/L NaF aqueous solution was added to the solution. After vigorous stirring for 1 h, the solution was transferred into a 100 mL of crystal glass reaction vessel, set for the autoclave, and heated at 160°C for 18 h with stirring in the sealed stainless cylinder. After that it was cooled down slowly to room temperature. Subsequently, the suspension was centrifuged at 8000 r/min for 10 min. The resultant product was then washed thoroughly and dried in vacuo. The product was annealed at 500 °C for 2 h to improve the crystallinity.

Annealing process of synthesized nanoparticles

After dried in vacuo, the synthesized nanoparticles were placed into crucible and put it into an oven. The temperature of oven took 2 h raised from room temperature to 500 °C and maintained at 500 °C for another 2 h. Then the oven was turned off and it took around 2 h to cool to room temperature from 500 °C naturally. The synthesized nanoparticles were collected to be used.

Synthesis of EDTA scaffold NaYF₄:Yb/Tm (20/1 mol%)

In a typical synthesis, 2 mL of 0.5 mol/L RECl₃ (RE = Y/Yb/Tm 79/20/1 mol%) aqueous solution and 1 mmol of EDTA were dispersed into 40 mL of deionized water and magnetically stirred for 1 h, forming a chelated RE-EDTA complex. Then 16 mL of 0.5 mol/L NaF aqueous solution was added to the solution. After vigorous stirring for 1 h, the solution was transferred into

a 100 mL of crystal glass reaction vessel, set for the autoclave, and heated at 160°C for 18 h with stirring in the sealed stainless cylinder. After that it was cooled down slowly to room temperature. Subsequently, the suspension was centrifuged at 8000 r/min for 10 min. The resultant product was then washed thoroughly and dried in vacuo. The product was annealed at 500 °C for 2 h to improve the crystallinity.

Synthesis of other metal doped NaYF₄:Yb/Er/x (18/2/2 mol%) x = Zn, Fe, Ni, Tm, V, Mo or Sm

In a typical synthesis, 2 mL of 0.5 mol/L RECl₃ (RE = Y/Yb/Er 80/18/2 mol% and 2 mol% of ZnCl₂, FeCl₃, NiCl₂, TmCl₃, VCl₃, MoCl₃ or SmCl₃) aqueous solution and 2 mmol of boric acid were dispersed into 40 mL of deionized water and magnetically stirred for 1 h, forming a chelated RE-B(OH)₃ complex. Then 16 mL of 0.5 mol/L NaF aqueous solution was added to the solution. After vigorous stirring for 1 h, the solution was transferred into a 100 mL of crystal glass reaction vessel, set for the autoclave, and heated at 160°C for 18 h with stirring in the sealed stainless cylinder. After that it was cooled down slowly to room temperature. Subsequently, the suspension was centrifuged at 8000 r/min for 10 min. The resultant product was then washed thoroughly and dried in vacuo. The product was annealed at 500 °C for 2 h to improve the crystallinity.

Chapter III

Synthesis of PEI-coated NaYF₄:Yb/Er/Gd (10/2/30 mol%) (LNP(Gd)-NH₂) and NaYF₄:Yb/Er (10/2 mol%) (LNP-NH₂)

The synthetic procedure followed that of F. Wang, et al.: in a typical experiment, to a well-stirred transparent solution of NaCl (210 mg, 3.6 mmol), YCl₃ (316 mg, 1.04 mmol), YbCl₃ (70 mg, 0.18 mmol), ErCl₃ (14 mg, 0.036 mmol), GdCl₃ (142 mg, 0.54 mmol), and PEI (0.60 g) in ethylene glycol (27 mL) was added a stoichiometric amount of NH₄F (266 mg, 7.2 mmol) in 18 mL of ethylene glycol. The resulting mixture was stirred for another 10 min, transferred to a 100 mL crystal glass reaction vessel, set for the autoclave, and then heated at 200°C for 2 h with

stirring in the sealed stainless cylinder. The obtained nanoparticles were collected by centrifugation, washed with ethanol and water several times, and dried in vacuo. The synthetic procedure of LNP-NH₂ was the same as above except for the amount of lanthanide ions: YCl₃ (479 mg, 1.58 mmol), YbCl₃ (70 mg, 0.18 mmol), and ErCl₃ (14 mg, 0.036 mmol).

Synthesis of mannose-coated NaYF₄:Yb/Er/Gd (10/2/30 mol%) (LNP(Gd)-GA-Man), NaYF₄:Yb/Er (10/2 mol%) (LNP-GA-Man), and galactose-coated NaYF₄:Yb/Er (10/2 mol%) (LNP-GA-Gal) using glutaric anhydride as a linker reagent

LNP(Gd)-NH₂ or LNP-NH₂ (20.2 mg) was dissolved in the solution of glutaric anhydride (12 mg, 0.1 mmol) and *N,N'*-diisopropylethylamine (DIPEA, 52 μ L, 0.3 mmol) in 4 mL DMF. After 2 h stirring at room temperature, the nanoparticles were collected by centrifugation, washed with DMF for several times, and dried in vacuo to give LNP(Gd)-GA or LNP-GA, which was dispersed in the solution of *N*-hydroxysuccinimide (NHS, 12 mg, 0.025 M) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 77 mg, 0.05 M) in 4 mL water. After 1 h stirring at room temperature, the nanoparticles were collected by centrifugation, washed with water for several times, and dried in vacuo. The nanoparticles thus obtained were added into the solution of 2-aminoethyl α -D-mannopyranoside^[30] (H₂N-Man, 90 mg, 0.125 M) in 4 mL water and reacted overnight at room temperature. The nanoparticles were collected by centrifugation, washed with water for several times, and dried in vacuo to give LNP(Gd)-GA-Man or LNP-GA-Man with mannose loadings of 4.0 ± 1.2 and 47 ± 3 μ mol/g, respectively. The synthetic procedure of LNP-GA-Gal was the same as above except for the use of 0.5 mmol 2-aminoethyl β -D-galactopyranoside (H₂N-Gal). LNP-GA-Gal contains 39 ± 5 μ mol/g galactose molecules.

Synthesis of COOH-coated NaYF₄:Yb/Er/Gd (18/2/30 mol%) (LNP(Gd)-COOH) and NaYF₄:Yb/Er (18/2 mol%)(LNP-COOH)

The synthetic procedure followed that of Z. Chen, et al.: a mixture of LNP(Gd)-OA or LNP-OA (100 mg), cyclohexane (100 mL), *t*-butyl alcohol (70 mL), water (10 mL) and 5% w/v K₂CO₃ aqueous solution (5 mL) was stirred at room temperature for ca 20 min. Then 20 mL aqueous

solution of Lemieux-von Rudloff reagent (5.7 mM KMnO_4 and 0.105 M NaIO_4) was added dropwise. The resulting mixture was stirred at ca 40 °C for over 48 h. The product was then isolated by centrifugation and washed with water and ethanol. Subsequently, the product was dispersed in hydrochloric acid (50 mL) of pH 4-5, the mixture being stirred for 30 min. At last, the oxidized product was obtained by centrifugation, washed twice with water, and dried in vacuo to give LNP(Gd)-COOH or LNP-COOH.

Synthesis of mannose-coated $\text{NaYF}_4\text{:Yb/Er/Gd}$ (18/2/30 mol%) (LNP(Gd)-CONH-Man), $\text{NaYF}_4\text{:Yb/Er}$ (18/2 mol%)(LNP-CONH-Man), and galactose-coated $\text{NaYF}_4\text{:Yb/Er/Gd}$ (18/2/30 mol%) (LNP(Gd)-CONH-Gal)

LNP(Gd)-COOH or LNP-COOH (100 mg) was dispersed in the solution of 4-dimethylaminopyridine (DMAP, 53 mg, 0.44 mmol) and *o*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU, 99 mg, 0.26 mmol) in water (2.8 mL). After an overnight reaction at room temperature, nanoparticles were collected by centrifugation, washed with water, and dried in vacuo. The nanoparticles thus obtained were added into 2.8 mL water containing 0.56 mmol $\text{H}_2\text{N-Man}$ and reacted at room temperature overnight. The nanoparticles were collected by centrifugation, washed with water, methanol, and chloroform for several times, and dried in vacuo to give LNP(Gd)-CONH-Man or LNP-CONH-Man with mannose loadings of 93 ± 0.5 and 56 ± 5 $\mu\text{mol/g}$, respectively. The synthetic procedure of LNP(Gd)-CONH-Gal was the same as above except for the use of 0.56 mmol 2-aminoethyl β -D-galactopyranoside ($\text{H}_2\text{N-Gal}$). LNP(Gd)-CONH-Gal contains 35 ± 3 $\mu\text{mol/g}$ galactose molecules.

Synthesis of mannose-coated $\text{NaYF}_4\text{:Yb/Er/Gd}$ (18/2/30 mol%)(LNP(Gd)-CONH-Man) with sonication

LNP(Gd)-COOH (25 mg) was dispersed in the solution of DMAP (13 mg, 0.109 mmol), DCC (13 mg, 0.065 mmol) in water (12.5 mL). After an overnight reaction at room temperature, nanoparticles were collected by centrifugation, washed with water, and dried in vacuo. The

nanoparticles thus obtained were added in 12.5 mL water containing H₂N-Man (25 mg, 0.139 mmol). After 5 h sonication at room temperature, nanoparticles were collected by centrifugation, washed with water, methanol, and chloroform for several times, and dried in vacuo to give LNP(Gd)-CONH-Man containing 196 ± 40 μ mol/g mannose molecules.

Phenol-sulfuric acid assay

Samples, standards, or control solutions were mixed with the solution of phenol (6% w/v) in water (1 mL). Then, 5 mL of concentrated sulfuric acid was added rapidly and directly to the solution so as not to contact the internal wall of the tube. The solutions were left undisturbed for 10 min before shaking vigorously. The absorbance at 490 nm was recorded after an additional 30 min.

ConA aggregation assay

ConA or BSA (final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 μ g/mL) was dissolved in the solution of NaCl (150 mM), CaCl₂ (5 mM), MgCl₂ (5 mM), MnCl₂ (5 mM), and DMSO (10%) in HEPES (10 mM, pH 6.8). LNP(Gd)-COOH (as control) or LNP(Gd)-CONH-Man was dispersed in HEPES (10 mM, pH 7.1) containing 10% DMSO with the final concentration of 1 mg/mL. In a 96-well microplate, each well of Lane A contained the LNP(Gd)-COOH solution (34 μ L) and the ConA solution (34 μ L) with the concentration varied. Likewise, Lane B is for LNP(Gd)-CONH-Man and ConA and Lane C is for LNP(Gd)-CONH-Man and BSA. At room temperature, the plate was left still for 1 h. The images of the post-assay microplates were taken from the bottom by a digital camera. Aggregation was quantified by turbidity of the sample in each well using the microplate reader described in the characterization section. For the NIR-irradiation assay, we conducted the aggregation experiments in a glass tube (1.5 mL) or microtube (0.5 mL) instead of a microplate well in a similar way as described for the microplate assay. The different parts of the microtube, i.e.; one on (“ON”) and another away from (“AF”) the aggregation, were irradiated with a focused laser incident light. LNP(Gd)-CONH-Man solution (0.5 mL) was mixed with ConA or BSA (as control) solution (0.5

mL) with the concentration varied in a glass tube. After the glass tube was left still for 1 h, the emission intensity of “AF” at 540 and 655 nm was measured. For “ON”, LNP(Gd)-CONH-Man solution (0.25 mL) was mixed with ConA or BSA (as control) solution (0.25 mL) with the concentration varied in a microtube. After the microtube was left still for 30 min, the emission intensities of “ON” at 540 and 655 nm were measured.

PNA aggregation assay

PNA or BSA (final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 µg/mL) was dissolved in the solution of NaCl (150 mM), CaCl₂ (5 mM), MgCl₂ (5 mM), MnCl₂ (5 mM), and DMSO (10%) in HEPES (10 mM, pH 6.8). LNP(Gd)-CONH-Gal was dispersed in HEPES (10 mM, pH 7.1) containing 10% DMSO with the final concentration of 1 mg/mL. LNP(Gd)-CONH-Gal solution (0.25 mL) was mixed with PNA or BSA (as control) solution (0.25 mL) with the concentration varied in a microtube. After the microtube was left still for 30 min, the emission intensities of aggregation at 540 and 655 nm were measured.

Macrophage aggregation assay

RAW 264.7, a mouse leukaemic monocyte macrophage cell-line, was gifted from SBI ALA Promo Co. Ltd. RPMI culture medium was purchased from RPMI Nacalai Tesque. LNP-GA-Gal or LNP-GA (final concentration: 2 mg/mL) dispersed in 50 µL RPMI culture media including 10 µL PBS buffer was mixed with 2×10^7 cells/mL 50 µL cell suspension (RAW 264.7) in RPMI culture media in a microtube. LNP-GA-Gal and LNP-GA (final concentration: 2 mg/mL) dispersed in 100 µL RPMI culture media including 10 µL PBS buffer was prepared as a negative control. After mixing the lysate by repetitive suction and ejection with a pipetter, it was transferred to a well of a 96-well flat-bottomed microplate and incubated for 24 h at 37 °C under 5% CO₂ atmosphere. The pictures of the cells were taken with a microscopy (×40). Both the supernatant and precipitate of LNP-GA-Gal+cell, LNP-GA+cell, LNP-GA-Gal+culture medium, and LNP-GA+culture medium were transferred to microtubes for the luminescent experiments. Photographs were taken with laser irradiation at 975 nm (1 W). The luminescence

spectra were measured with the laser light (980 nm, 0.3 W) focused on the aggregation.

Chapter IV

MTT assay of LNPs

MKN45 cell, a cancer cell line from stomach cancer of human, was gift to S. Ogura from Dr. Suzuki (Fukushima Medical College, Fukushima, Japan). Culture medium (RPMI-1640, FBS (+), ABAM (+)) of MKN45 cell was taken out from a 10 cm dish and the cells were washed twice with 2 mL PBS solution. After that, 1 mL of trypsin was added and incubated with cells at 37 °C in an atmosphere of 5% CO₂ for 5 min. After 5 min, 5 mL of fresh culture medium was added in order to quench. Total 6 mL solution containing cells were centrifugated at 1100 rpm for 5 min. Before centrifugation, 10 µL samples from 6 mL solution was mixed with trypan blue, which is a vital stain used to selectively color dead tissues or cells blue, and the numbers of living cells were counted with hemocytometer. After centrifugation, supernatant was replaced with 2 mL fresh culture medium. The cells were placed at a cell density of 2×10^4 cells/well into 96 well plates, 100 µL for each well. After cultured overnight, the cell medium was replaced with the test LNPs solution at different concentrations (in culture medium) and cultured overnight. Then, the LNPs solutions were removed and the cells were washed with PBS solution for 100 µL/well. 100 µL fresh culture medium and 10 µL MTT methanol solution (diluted 10 times by PBS solution) was added to each well. After 4 h of incubation, 100 µL/well of 10% SDS solution was added. After overnight incubation, a microplate reader model 550 was used to measure the absorbance of each well at 570-660 nm. The percentage of cell viability for each sample was calculated by comparing the absorbance with 100% viability samples (PBS buffer as positive control) and 0% viability samples (SDS as negative control).

LNPs in PDT experiment

MKN45 cell, a cancer cell line from stomach cancer of human, was gift to S. Ogura from Dr. Suzuki (Fukushima Medical College, Fukushima, Japan). Culture medium (RPMI-1640, FBS (+),

ABAM (+) of MKN45 cell was taken out from a 10 cm dish and the cells were washed twice with 2 mL PBS solution. After that, 1 mL of trypsin was added and incubated with cells at 37 °C in an atmosphere of 5% CO₂ for 5 min. After 5 min, 5 mL of fresh culture medium was added in order to quench. Total 6 mL solution containing cells were centrifugated at 1100 rpm for 5 min. Before centrifugation, 10 µL samples from 6 mL solution was mixed with trypan blue, which is a vital stain used to selectively color dead tissues or cells blue, and the numbers of living cells were counted with hemocytometer. After centrifugation, supernatant was replaced with 2 mL fresh culture medium. The cells were placed at a cell density of 2×10^4 cells/well into 96 well plates, 100 µL for each well. After cultured overnight, the cell medium was replaced with 5-aminolevulinic acid (ALA) at different concentrations (in culture medium) together with 2 mg/mL of tested LNPs and cultured for 24 h. Then, the plates were irradiated under NIR laser (980 nm, 400 mA) 50 mm above for 90 min. And then the LNPs solutions were removed and the cells were washed with PBS solution for 100 µL/well. 100 µL fresh culture medium and 10 µL MTT methanol solution (diluted 10 times by PBS solution) was added to each well. After 4 h of incubation, 100 µL/well of 10% SDS solution was added. After overnight incubation, a microplate reader model 550 was used to measure the absorbance of each well at 570-660 nm.

Acknowledgements

The research in this thesis was carried out under the direction of Professor Hideya Yuasa at the Department of Life Science, Graduated School of Bioscience and Biotechnology, Tokyo Institute of Technology.

This work was supported by a “Collaborative Research Based on Industrial Demand” program (to H.Y., S.O., Y.M., and E.O.) from Japan Science and Technology Agency (JST) and a Grant-in-Aid for Scientific Research (No. 24710248 to A.S.) from the Japan Society for the Promotion of Science (JSPS). We are also grateful to SBI Pharmaceuticals Co., Ltd. for the financial support.

The author would like to express his greatest thanks to Professor Hideya Yuasa for his kindness, sufferance, continuous guidance, helpful advice, and warm encouragement throughout this study. The author is also grateful to Dr. Atsushi Shimoyama for his wise suggestions.

The author is thankful to Prof. Hisakazu Mihara and Miss Saki Matsumoto at Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, for their assistance in the dynamic light scattering (DLS) analyses. I would like to thank Mr Hori Katsuaki and Mr Yuichi Suzuki, Center for Advanced Material Analysis, National Corporation Tokyo Institute of Technology, for TEM and XRD measurements. I appreciate Dr. Motowo Nakajima of SBI ALA promo Co. Ltd for the gift of RAW264.7 and Dr. Suzuki of Fukushima Medical College, Fukushima, Japan for the gift of MKN 45.

The author also would like to thank Miss Izumi Sotokawa and Mr Takuma Ikejiri for their assistance in doing experiments and their advice, and all members in Yuasa Laboratory for their kindness and valuable discussions.

Finally, the author would like to thank his family, Liu Zhijie, Long Gangjian and Lee for their support and love.