Project:

Preliminary studies on beta-cell mass quantification by using ^{13}C MR technique

&

Dosage investigation on NIR agent indocyanine green by using UV-vis spectroscopy

Prepared and submitted by:

Qiuju Zhou

Period: 15/02/2014 - 15/08/2014

Part 1:

Preliminary studies on beta-cell mass quantification by using ^{13}C MR technique

1.1 Purpose

It is reported that patients with type II diabetes only possess half of their pancreatic beta-cell mass at autopsy [1]. This finding leads to lots of researchers paying close attention to seeking method for exact monitoring the fate of beta-cells under disease and therapy conditions in the hope of a better understanding of diabetes pathogenesis. While, the fact that millions of beta-cells, which only comprise 1% of pancreas total weight and are discrete structures scattered throughout the pancreas challenges in vivo beta-cell imaging dramatically[1]. It is reported that D-mannoheptulose can be transported into beta-cells at the intervention of GLUT2 (Glucose transporter 2)[2] and thus can be used as a tool to label preferentially insulin-producing cells in the pancreatic gland in the perspective of the non-invasive imaging of the endocrine pancreas [3]. Here, in order to check the possibility of in vivo ^{13}C MR study on beta-cell mass quantification with the marker of D-mannoheptulose, the effect of intravenous infusion of carbon-13 enriched D-mannoheptulose in rat and of ingested mannoheptulose in human have been investigated by using ^{13}C MR technique.

1.2 Materials and methods

1.2.1 Rat tissues (section 1)

Seven kinds of rat tissue samples (details were listed in table 1 and table 2): 1) liver (Abbr. LV), 2)intestine (Abbr. IN), 3)kidney (Abbr. KID), 4)brain (Abbr. BR), 5)pancreas (Abbr. PAN), 6)red cells (Abbr. GR), and 7)plasma ((Abbr. PLS1) were provided by the research group of Prof. Willy J. Malaisse. Each tissue has 6 samples: one control sample (marked with number 2) was taken from animals injected with saline only and the other five samples were taken from animals, 30, 30, 60, 90, and 180 minutes after the injection of C-13 mannoheptulose (marked with number 3, 4, 5, 8, and 10, respectively).

Table 1. weight of tissues.

rat	Time/min	Body Weight(g)	Intestine (g)	Liver (g)	Brain (g)	Pancreas (g)	Kidney (g)	
	X(ctrl)	288	0.38	12.24	1.71	1.13	1.09	1.08
2	30	240	0.51	8.15	1.75	1.41	1.1	1.14
3	30	230	0.37	8.48	1.56	0.91	0.95	0.92
4	60	215	0.5	7.66	1.65	1.03	0.94	0.87
5	90	258	0.36	9.36	1.43	0.92	1.01	0.94
6	180	285	0.38	10.28	1.7	1.44	1.07	1.06

Table 2. Numbers of samples.

rat	Time/min	Intestine	Liver	Brain	Pancreas	Kidney	Plasma	Red cells
1	X(ctrl)	2IN	2LV	2BR	2PAN	2KID	2PLS1	2GR
2	30	3IN	3LV	3BR	3PAN	3KID	3PLS1	3GR
3	30	4IN	4LV	4BR	4PAN	4KID(2kidneys)	4PLS1	4GR
4	60	5IN	5LV	5BR	5PAN	5KID	5PLS1	5GR
5	90	8IN	8LV	8BR	8PAN	8KID	8PLS1	8GR
6	180	10IN	10LV	10BR	10PAN	10KID	10PLS1	10GR

1.2.2 Rat tissues (section 2)

Two male Wistar Han rats (one, 430 grams, was used as control and injected with 1000 µl PBS, the other, 512 grams, was injected with 80 mg D-[1-^{13}C]-mannoheptulose dissolved in 1000 µl PBS) were placed into box and anesthetized with isoflurane during weighting process. Subsequently, they were anesthetized with 460 µl mixed solution of Rompun and Ketamine [Rompun (2%, 20mg/ml) 60 µl, Ketamine (Ketamine1000, 100mg/ml) 400 µl] and then tail vein injection was performed. 30 minutes after the injection, the rat was euthanized by carbon dioxide. Seven samples (blood, liver, pancreas, intestine, kidney, urine and brain) were dissected out.

1.2.3 Volunteer urine sample

One healthy 35-year old female volunteer without a family history of diabetes mellitus fasted overnight. At 8:00, she had breakfast and at 11:00 she consumed 212 or 177 grams sliced avocado. The experiments began at 10:30 a.m., when the fruit was ingested. Following this, 2 hour specimens of urine were collected up to the end of 12th hour. At 14:00, she had lunch and at 18:00, she had supper. Urine samples were centrifuged and 500 µl supernatant were transferred into 5mm NMR tube for ^{13}C NMR test. All the procedures were done at room temperature (ca. 298K).

1.2.4 MR experiments

The spectra were recorded on an Avance 300 spectrometer (Bruker, Karlsruhe, Germany) with a 7.4 superconducting magnet. The ^{13}C NMR spectra of rat tissues (brain, red cells, intestine, liver, kidney, and pancreas) were performed with a 10 mm BBI probe at a frequency of 75.5 MHz using a standard “zgpg30” sequence and the D1 value and scan number were fixed at 2 seconds and 1024, respectively. The temperature of the sample was regulated at 278 K by a Bruker BVT-2000 unit.

Spectra of liver, kidney, intestine, brain, pancreas, and red cells were referenced to an external solution of methanol (150 µl, HPLC grade). The height of the tested samples was kept around 2 cm (ca. 2 mL). Except liver, the other samples were replenished with 0.20 M phosphate buffer saline solution (PBS: NaH_2PO_4-K_2HPO_4 with pH 7.40) to ensure the tested height.

Spectra of plasma were referenced to an external solution (60 µl methanol mixed with 1500 µl PBS) in a 10 mm NMR tube. The volume of plasma was kept at 500 µl in a 5 mm NMR tube.

For urine samples, a 5 mm BBI probe was used and 40 µl methanol was used as an internal reference and the test was perform under room temperature.

1.3 Results and discussions

In our study, it is the carbon atom at position 1 of D-mannoheptulose which is labelled with ^{13}C isotope, i.e. D-[1-^{13}C]-mannoheptulose, and this carbon atom gives a resonance around 64.8 ppm.

1.3.1 Rat tissues (Section 1)

Theoretically, in the ^{13}C NMR spectra of samples containing D-[1-^{13}C]-mannoheptulose should give a resonance around 64.8 ppm and of samples collected after the injection of D-[1-^{13}C]-mannoheptulose, the intensity of this resonance should show a trend of first increase then decrease with the increase of the injection time. For the spectra of controls, which do not containing D-[1-^{13}C]-mannoheptulose, normally, there should be no resonance around 64.8 ppm. For the two samples which were taken from animals 30 minutes after the injection, if there is a peak around 64.8 ppm, the intensity of that signal should be equal to each other.

To interpret the spectra, we mainly focus on two resonances: δ=49.50 ppm, signal of the reference signal and δ=64.8 ppm, signal of D-[1-^{13}C]-mannoheptulose. For all the samples, the reference signal (δ=49.50 ppm) can be observed but the intensity and the shape of this resonance are not identical due mainly to the tissue susceptibility and the disturbance of the resonances come from the sample itself, and also due to the slightly difference in tested height.

In the spectra of the brains, there is no signal around 64.8 ppm. These results can be explained that brain does not contain D-[1-^{13}C]-mannoheptulose.

In the spectra of intestine, red cells, and pancreas, the observed results were contradictory to the facts. For example, 1) 2GR does not contain D-[1-^{13}C]-mannoheptulose but the typical resonance of this compound appears in the spectra; 2) 3GR, an analogue sample of 4GR, does not give a peak around 64.8 ppm as 4GR; 3) 4IN, an analogue sample of 3IN, does not give a peak around 64.8 ppm as 3IN; 4) 4PAN, an analogue sample of 3PAN, does not give a peak around 64.8 ppm as 3PAN.

In the spectra of plasma (Figure 1.1), sharp peak (δ=64.8 ppm) was observed in the spectra of 2PLS1, 3PLS1, 4PLS1, and 5PLS1. If just considering the five samples taken from animals 30, 30, 60, 90, 180 minutes after the injection of D-[1-^{13}C]-mannoheptulose, this group of spectra seems reasonable because the intensity of D-[1-^{13}C]-mannoheptulose shows a trend of first increase then decrease with the increase of the injection time. But, the sharp peak was also observed in the spectrum of the control sample, 2PLS1, which was contradictory to the facts: 2PLS1 does not contain D-[1-^{13}C]-mannoheptulose. The same results were observed with kidney tissues.

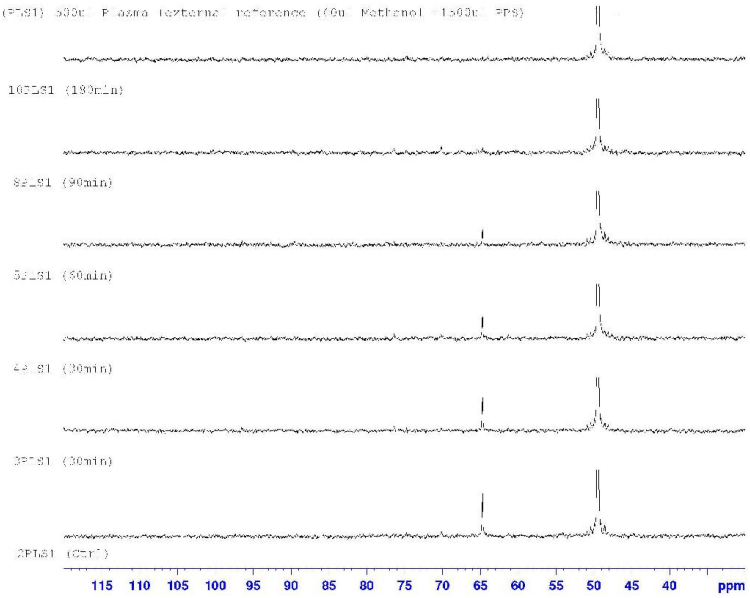

Figure 1.1. The ^{13}C NMR spectra of rat plasma samples.

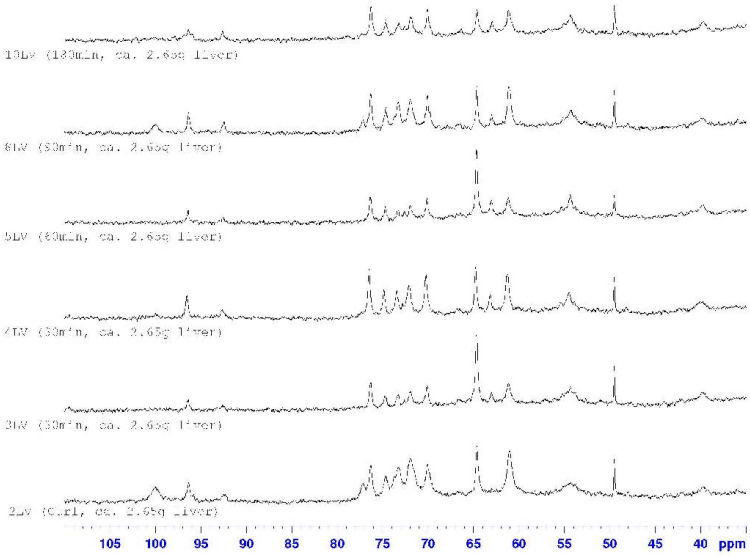

Figure 1.2. The ^{13}C NMR spectra of rat liver samples.

In the spectra of livers (Figure 1.2), the sharp peak (δ=64.8 ppm) was observed in the spectra of all six samples and the intensity of this resonance roughly shows a trend of first increase then decrease with the increase of the injection time, except sample 4LV. Moreover, the sharp peak was also observed in the spectra of the control sample, 2LV, which was contradictory to the facts: 2LV does not contain D-[1-^{13}C]-mannoheptulose. Interesting, there is a new signal (δ=63 ppm) occurs in the spectra of the samples taken from animals after the injection of D-[1-^{13}C]-mannoheptulose, compared with the control sample, 2LV. However, the intensity of this new resonance (δ=63 ppm) does not show a trend of first increase then decrease with the increase of the injection time.

1.3.2 Rat tissues (Section 2)

The preliminary results obtained with rat tissues (section 1) did not give convincing information about the evolution of D-[1-^{13}C]-mannoheptulose at different tissues varies after injection time. In order to find the cause for the contradictory results, the second section of rat tissues were prepared and tested.

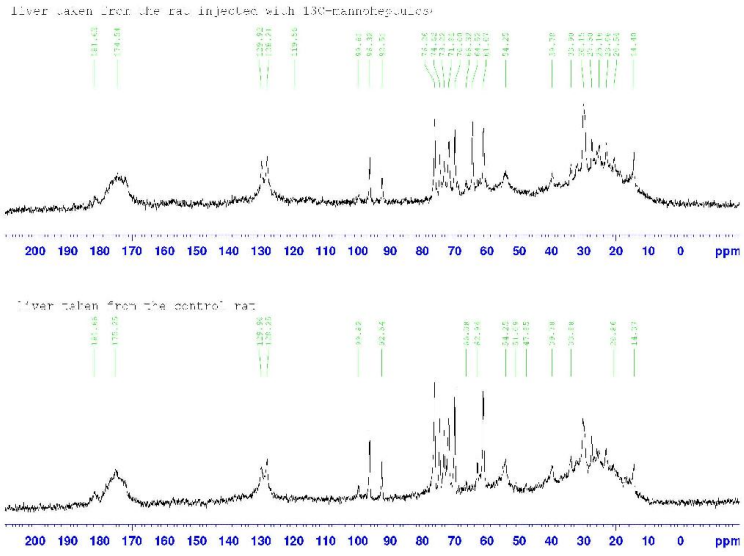

Figure 1.3. The ^{13}C NMR spectra of rat liver samples.

As shown in Figure 1.3, a sharp peak (δ=64.8 ppm) was only observed in the spectrum of the liver tissue taken from the rat injected with D-[1-^{13}C]-mannoheptulose. Similar results were also obtained with rat kidneys, blood, and urine, which indicating wrong numbering of rats in sample preparation caused the contradictory results for the first section of rat tissues.

In addition, a resonance around 100 ppm belongs to the glycogen-C1 resonance, is observed. In the spectrum of the liver taken out from the control rat, the intensity of this peak is much higher, indicating of deposition of glycogen probably caused by D-mannoheptulose.

In the spectra of urine (see Figure 1.4), the resonances of the other six carbon atoms of D-[1-^{13}C]-mannoheptulose can also be observed. These results indicate that the major portion of D-[1-^{13}C]-mannoheptulose is excreted in the urine and a small amount still remains in blood, liver, and kidney 30 minutes after injection. Moreover, in comparison with the spectrum of a mixture solution containing glucose and D-[1-^{13}C]-mannoheptulose, we found that the other signals in the spectrum of urine collected from the rat injected with D-[1-^{13}C]-mannoheptulose belong to glucose. This result indicated that the injection of D-[1-^{13}C]-mannoheptulose into rat did arise glucosuria.

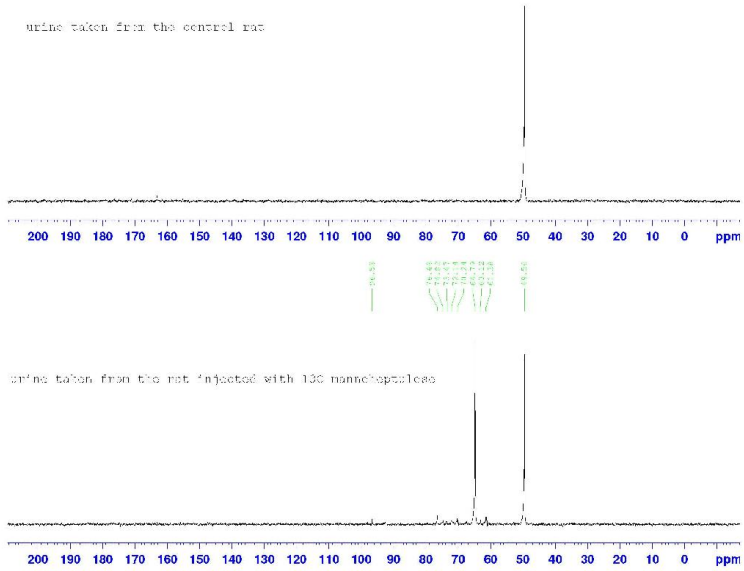

Figure 1.4. The ^{13}C NMR spectra of rat urine samples.

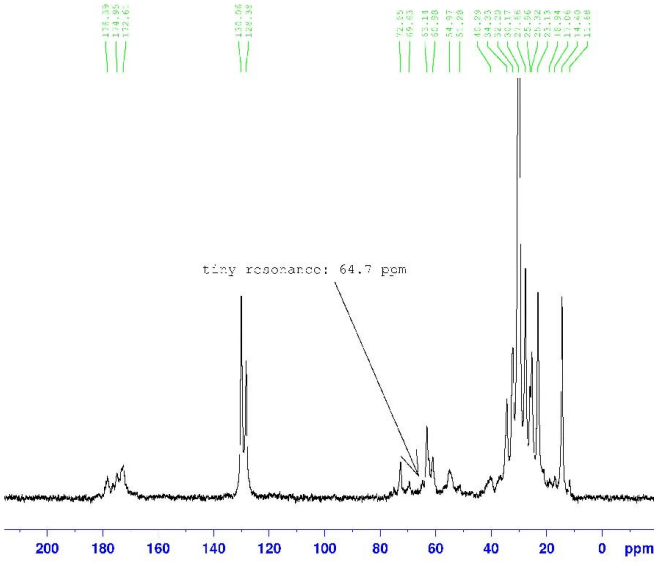

Figure 1.5. The ^{13}C NMR spectrum of rat pancreas (taken from the rat injected with D-[1-^{13}C]-mannoheptulose and the major portion of fat has been removed by using Soxhlet extractor).

In the spectrum of pancreas (see figure 1.5), the resonance around 64.8 ppm can not be observed easily, even after the removal of major portion of fat. This result is, somewhat, in contradiction with the statement that D-mannoheptulose can be transported into cells at the intervention of GLUT2 because pancreatic beta-cells contain GLUT 2. While this contrary result might be due to the small mass of beta-cells in pancreas.

1.3.3 Volunteer urine sample

Our results show that mannoheptulose appears in the urine of normal person after the uptake of avocado fruit. The peak of excretion of mannoheptulose occurs in the 2 to 4 hour period after the ingestion of avocado. The rate of mannoheptulose excretion begins to drecrease 6 hours after consumption of this sugar. It is reported the content of D-mannoheptulose in avocado fruit ranges between 0.1 to 3.4% of dry weight. Approximately over 50% of the ingested mannoheptulose appears in the urine, while several researchers had reported that less than 20% of the ingested mannoheptulose appears in the urine by using Dische's orcinol reaction.

1.4 Conclusions

Our preliminary results show that ^{13}C NMR is a straightforward method, 1) to check the distribution and the evolution of D-[1-^{13}C]-mannoheptulose at different rat tissues; 2) to observe deposition of glycogen and glucosuria followed by D-mannoheptulose administration. No obvious evidence for hyperglycemia was observed perhaps because of the short acquisition time or the low concentration of blood [glucose] in rats (2.8-7.5 mM). In addition, ^{13}C NMR is a convenient tool for study the effect of ingested mannoheptulose in human.

1.5 References

1. Paulev-Zubieta, New Human Physiology, 2nd Edition, http://www.zuniv.net/physiology/book/index.htm
2. H. G. Coore, P. J. Randle, Ernst Simon, Peretz F. Kraicer, M. C. Shelesnyak, Block of insulin secretion from the pancreas by D-mannoheptulose, Nature, 1963(197) 1264-1266.
3. Willy J. Malaisse, Karim Louchami, Abdullah Sener, Noninvasive imaging of pancreatic beta cells, Nat. Rev. Endocrinol, 2009 (5) 394-400.
4. Donald W. McRobbie, Elizabeth A. Moore, Martin J. Graves, Martin R. Prince, MRI from Picture to Proton, 2nd Edition, Cambridge University Press, 2006.
5. Robin A. De Graaf, In Vivo NMR Spectroscopy, 2nd Edition, John Wiley & Sons, Ltd, 2007.

Part 2:

Dosage investigation on NIR agent indocyanine green by using UV-vis spectroscopy

2.1 Introduction

Indocyanine green, currently the only ICG fluorophore that is registed with the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for clinical application, has a peak spectral absorption in the range of infrared (ca. 800 nm) allowing its diagnosis value for determining cardiac output, hepatic function, and liver blood flow, and for ophthalmic angiography [1]. However, the important factors such as dose and timing of ICG, for clinical applications, still remain contentious. For example, Hutteman et al.[2] reported that no difference was observed between the 5-mg and 10-mg groups, while Gilmore et al. [3] concluded that, as the dose of ICG was increased, the signal-to-background ratio increased from a median of 3.1 to 8.4 to 10.9 over the range of 100, 250, and 500 μM, respectively.

Here, in order to provide information for explaning this contentious conclusions, we investigated the spectral properties of ICG-Pulsion in water and in aqueous Human Serum Albumin (HSA) solution by using UV-vis spectrocopy. Our premilinary results show that monomer molecule of ICG-Pulsion is the major contributor to NIR signal intensity of ICG-Pulsion and can be captured by HSA and therefore shows a high photostability and thermal stability in aqueous HSA solution. When conducting dosage investigation, surely the most sensible thing would be to dissolve ICG-Pulsion in HSA aqueous solution and the final molar ratio of HSA to ICG should be fixed around 13%. To analysis clinical results should be directed against specific conditions and consider more factors, such as EPR effect.

2.2 Materials and methods

Human serum albumin, 96 – 99% purity (catalogue no. A-1653) and ICG-Pulsion were purchased from Sigma (Bornem, Belgium) and used without further purification. Double distilled water was used thoughout the experiments. All the absorption spectra (from 1100 nm to 500 nm) were recorded on a Perkin-Elmer UV-vis spectrometer and a 10mm quartz cuvette. The stock solution of HSA (50 μM, 15ml) and of ICG-Pulsion (6.4 mM, 1ml) were freshly prepared by directly dissolving it in distilled water and stored in dark at 277 K. The samples used in UV tests were prepared by diluting the above stock solutions to the required concentration with double distilled water.

2.3 Results and Discussions

Figure 2.1 shows the degradation of 7.5 μM ICG-Pulsion monomer (λ_{max} ca. 780 nm) in water solution with exposure time. If the concentration of ICG-Pulsion is lower than 15 μM, about 8-hour after the preparation, most of the monomers convert into leucoform and oligomer. Subsequently, dimer (λ_{max} ca. 680 nm) can be formed. If the concentration of ICG-Pulsion is higher than 15 μM and stored in dark at 277 K, about 36-hour after the preparation, J-aggregates (λ_{max} ca. 896 nm) can be formed.

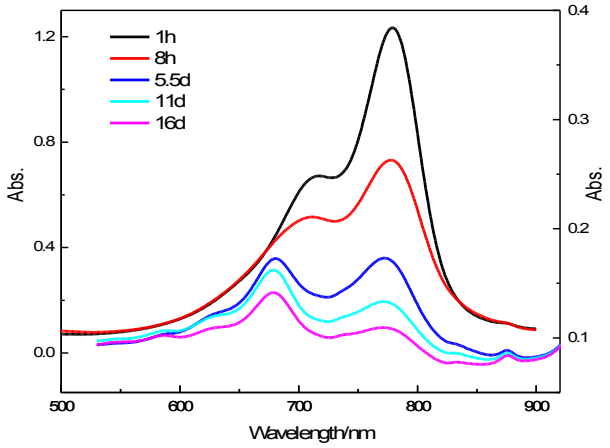

Figure 2.1 The degradation of 7.5 μM ICG-Pulsion aqueous solution with exposure time.

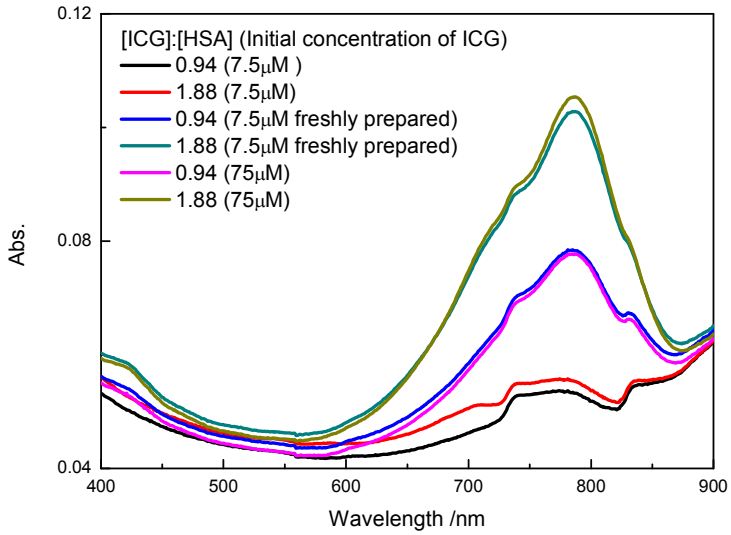

Figure 2.2 The effects of the introdution of HSA on the aborption profile of different ICG-Pulsion stock solution.

In two-hour after the preparation, the addition of HSA can not only prevent the degradation of monomer but also recover the degraded monomers. As shown in Figure 2.2, the addition of HSA into two sets of 7.5 μM ICG-Pulsion solution (both were the dilution of 75μM ICG-Pulsion) leads to different absorption profile. The

addition of HSA into the freshly prepared 7.5 μM ICG-Pulsion solution gives an identical absorption profile as 75 μM ICG-Pulsion. While the addition of HSA into the older solution (exposed for 24 hours) can not recover the degraded monomer and leads to a very weak absorption signal. So in a short period after the preparation of ICG-Pulsion solution, the degraded monomer can be converted into monomer in the presence of HSA. Figure 2.3 shows the effects of [ICG] to [HSA] ratio on the content of monomer in HSA aqueous solution. As shown in figure 2.3, when the [ICG] to [HSA] ratio is around 13%, in the solution, the monomeric absorption is dominant.

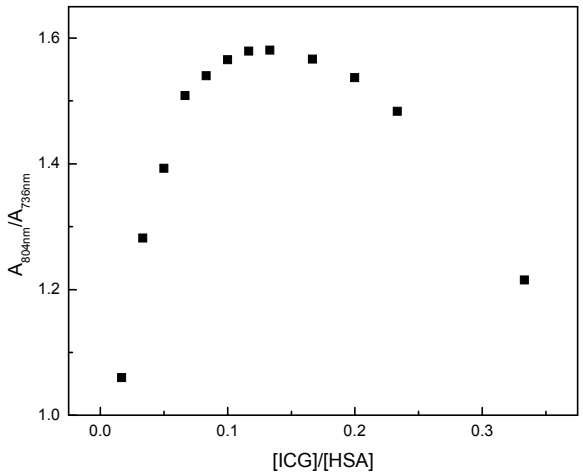

Figure 2.3 The effects of [ICG]/[HSA] on the portion of monomer in HSA aqueous solution.

2.4 Conclusions

Our premilinary results show that monomer molecule of ICG-Pulsion is the major contributor to NIR signal intensity of ICG-Pulsion and can be captured by HSA and therefore shows a high photostability and thermal stability in aqueous HSA solution. Therefore, when investigating the dosage and timing of ICG-Pulsion in clinical applications, it would be better to dissolve ICG-Pulsion in HSA aqueous solution and the final molar ratio of HSA to ICG should be fixed around 13%. To analysis clinical results should be directed against specific conditions and consider more factors, such as EPR effect.

2.5 References:

[1] N. Kokudo; T. Ishizawa, Fluorescent Imaging : Treatment of hepatobiliary and pancreatic diseases,（Frontiers of gastrointestinal research, v. 31）Karger, c2013

[2] Hutteman, M., J. R. van der Vorst, et al. (2011). "Near-infrared fluorescence imaging in patients undergoing pancreaticoduodenectomy." Eur Surg Res **47**(2): 90-97.

[3] Gilmore, D. M., O. V. Khullar, et al. (2013). "Effective low-dose escalation of indocyanine green for near-infrared fluorescent sentinel lymph node mapping in melanoma." Ann Surg Oncol **20**(7): 2357-2363.

Symposiums, workshops and training

Bruker BeNeLux NMR User meeting (Brussels, Belgium, 15/11/2013)

Acknowledgement

I would like to thank Prof. Robert N. Muller and Prof. Luce Vander Elst for their many insightful comments and powerful supports. I would like to thank Dr. Sophie Laurent, Dr. Celine Henoumont, Dr. Sebastien Boutry, Dr. Carmen Burtea, Dr. Lionel Larbanoix, Mathieu Roch for many fruitful discussions and technical assistance. I would like to thank Prof. Willy J. Malaisse and Dr. Karim Louchami for many valuable discussions about the challenge in beta-cell quantification and providing rat tissues. I would like to thank Mrs. Patricia de Francisco for her help in preparing manuscript. This work was performed with the financial support of the BELSPO grant.