

BITERIALS Co., Ltd



Technology Overview

1. Background of Technology

Nanotechnology can be defined as "Scientific technology of understanding and controlling materials in nanometer size." Nanotechnology is regarded as one of the most powerful technology to determine the level of national scientific technology that can lead the continuous progresses of industry in 21th century with IT, BT and CT as shown in the Scheme 1. In present, nanotechnology is in the starting point and the governments in the world are tremendously investing its R&D areas. Thus, the market of products in nanotechnology is being estimated more than a billion dollar and thus, the future of nanotechnology is expected to be very hopeful.¹



Scheme 1. (a) Market application focus of nanotechnology companies in America (b) Market application focus of nanotechnology companies in Europe

0		worldwi	Expected market share		
Scenario	Related markets	2006 (billion won)	2020 (billion won)	(standard of 2020)	
Early diagnosis of Disease and	Nano-bio Chip for spot diagnosis (Lab-on-a-Chip)	44	23,610	40 %	
diagnosis using Nano-biotechnology	Implant nano-biosensor for diagnosis	-	10,500	10 %	
Through development of limited control	Nano composite microscope	-	1,500	40 %	
monomer and single cell level is possible	Single molecule DNA and protein searcher	-	15,000	60 %	

Table	1. Market	demand o	f the	nano-biomedical	diagnosis	& therapy
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Nanotechnology is being applied for various areas like semiconductors and nanobiomaterials. *In vivo* molecular image materials, bio sensor or medicinal materials are incredibly developed from the progress of nanomedical technology expecting the early diagnosis of disease and activation of self-diagnosis. Thus, in the nanomedical area the concept of theragnosis was born indicating a method for diagnosis and therapy at the same time. Nanomedical technology is started form America and Europe and increased continuously to the world. Market demands of the nano-biomedical application are expected as shown in Table 2.

Medicines developed recently are mostly replacing the used ones of specifically targeted medicines for specific diseases. The progress of new therapies using targeted drug delivery system and non-invasive *in vivo* imaging are opening the hopeful future in the nanomedical diagnosis and therapy for extension of life as seen in the Table 2 about market demand in the world.²

Scenario	Related	worldwide	e market	Expected market share
	markets	2006 (billion won)	2020 (billion won)	(standard of 2020)
Extension of life and induction of revolution in medical part through page	Nano-material for drug delivery	4,000	32,500	60 %
biomedical treatment technology of self- assembled, specific disease targeted, minimum stress and topical papo-bio	Nano- biomedical instrument	22,000	42,000	30 %
treatment for extension of life	Nano-material for cell therapy and tissue engineering	100	22,450	30 %

Table 2. Market demands in the treatment of intractable diseases



Especially PET and MR being used from several decades ago are very useful for finding position or range of cancers and so determining their transition status. However, these methods could not provide the real-time intraoperative visualization of disease processes. Thus, the surgeon could not remove the lymph node from the normal organs and thereby make the risk of lymphedema. Sentinel lymph node imaging is commonly performed prior to surgery for breast cancer and melanoma using gamma probe. In breast cancer surgery SLN biopsy by nuclear active material has become a standard of care and recently this method starts to apply for finding transition pass ways in the case of stomach cancer and abdominal cavity. However, it is difficult to follow up the transition of cancer in the abdominal cavity because there are so many pass ways. The gamma probe could not be used with much amount because of harmful materials to body. So, instead of a radioactive probe it was reported that fluorescence of quantum dot was used for SLN biopsy in the real time surgery.³ However, quantum dots have limitation to be applied for clinical experiment because of original toxicity. Finally it is necessary to develop a safe material to image non-invasively.

2. Description on Technology Applied

1. Synthesis of multifunctional nanoparticles (MNP-SiO₂(dyes)) for PET/ MR/Optical image

To control of size of magnetic nanoparticles micro emulsion method was used. We obtained 5 ~ 15 nm sized magnetic nanoparticles and total size including silica and dye above 30 nm. The procedure of synthesis was shown in scheme 2. Magnetic core of $CoFe_2O_4$ was synthesized by refluxing ethanol solutions of Co^{2+} and Fe^{3+} and sodium dodecylbenzene sulfonate in the xylene with NH₂NH₂. Next, according to Stöber process, the $CoFe_2O_4$ nanoparticles coated by polypyrrolidone for solubility in ethanol, tetraethyl orthosilicate (TEOS) and fluorescent organic dye modified by triethoxysilane group are mixed with NH₄OH at room temperature.



Scheme 2. Synthesis of fluorescent magnetic nanoparticles, MNP-SiO₂(dyes)



2. Properties of multifunctional nanoparticles for PET/ MR/Optical image

2-1. Size

The transmission electron microscopy (TEM) images of fluorescent magnetic silica nanoparticles are shown in Figure 1. According to the fluorescent organic dyes, (a), (b), (c) and (d) are nanoparticles which have Cy-648, NIR-730, Cy-749 and NIR-797 respectively. The sizes of core in (a) and (c) are 5 nm and those in (b) and (d) are 15 nm. The total size is about 45 nm.



Figure 1. TEM images of fluorescent magnetic silica-coated nanoparticles (MNP- $SiO_2(dyes)$). (a) , (b), (c) and (d) are nanoparticles which have Cy-648, NIR-730, Cy-749 and NIR-797 respectively. The sizes of nanoparticles are about 45 nm and the scale bar in the images is 100 nm.

2-2. Emission spectra

The emission spectrum of MNP-SiO₂(dyes) nanoparticles which have Cy-648, NIR-730, Cy-749 and NIR-797 in the PBS, pH 7.4 showed maximum wavelength at 675, 760, 775 and 830 nm in the Figure 2.



Figure 2. Emission spectra of MNP-SiO₂(dyes) in PBS, pH 7.4



2-3. T2 relaxivity coefficient (r2)

The proton T2 relaxivity of MNP-SiO₂(NIR797) nanoparticles was determined by measuring the change in T2 at increasing concentrations of nanoparticles using a 4.7 T MRI system (Bruker Instrument). The transverse relaxivity (r2) was calculated as 95 and 61 s⁻¹ per mM of Co and Fe. (a) is a line for 15 nm magnetic core in the MNP-SiO₂(NIR797) and (b) is one for 5 nm magnetic core in the MNP-SiO₂(NIR797). This value was found to be 20 times more than that of commercially available Gd-HP-DO3 A, 4.8 s⁻¹ per mM.



Figure 3. r2 relaxivity curve of (a) 15 and (b) 5 nm of magnetic core in the MNP-SiO₂(dyes) nanoparticles.

2-4. Cell viability by MTT assay

3(4,5-dimenthylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) cell-viability assay showed that MNP-SiO₂(NIR797) nanoparticles were not toxic to HeLa cell (Human epithelial carcinoma cell line) as shown in Figure 4. They were completely viable even after incubation with MNP-SiO₂(NIR797) nanoparticles loading as high as 0.8 mg/ per 5000 cells (58.2 μ g /mL Co+Fe) for three days.



Concentration (mg/mL)

Figure 4. Viability of cells by MTT assays of HeLa cell incubated with different amounts of MNP-SiO₂(NIR797) nanoparticles for 24, 48 and 72 hours. The results are represented as a percentage of absorbance relative to control cells (100 %, nanoparticles free medium). The data are expressed as mean \pm SD of three independent experiments



2-5. Optical images

We injected 2 mg kg⁻¹ of MNP-SiO₂(NIR797) nanoparticles (equal to 145 mg kg⁻¹(Co+Fe)) subcutaneously in the left forepaw of the mouse (6 weeks) for optical imaging as shown in Figure 5(a) and (b) using spectral imaging system (Maestro In-Vivo imaging System, CRI Inc., Woburn, MA). The axillary lymph node was clearly visualized through the skin at 5 minute post injection in the Figure 5(c).



Figure 5. MNP-SiO₂(NIR797) nanoparticles sentinel lymph node mapping in the mouse. *In vivo* images of mouse injected subcutaneously with 2 mg/kg of NPs (145 mg/kg (Co+Fe)) right after post injection, (a) and (b) and at 5 minute post injection (c). Images were obtained by overlaying an image under white light with that of NIR fluorescence.

We injected 1 x 10^6 A549 cells labeled by MNP-SiO₂(NIR797) nanoparticles in the liver of mouse (7 weeks) as surgery as shown in the Figure 6(a) and took fluorescence image, Figure 6(b) from the outside of the mouse to validate whether the cells are visible or not without surgery. Figure 6(c) is a image of merge of (a) and (b)



Figure 6. Fluorescence image of liver injected by A549 cells which are labeled by MNP- $SiO_2(NIR797)$ nanoparticles

2-6. PET/MR images



For the attachment of positron emitter, ⁶⁸Ga ($t_{1/2} = 2 h$), MNP-SiO₂(NIR797) nanoparticles were first coated with 3-aminopropyltriethoxysilane and conjugated with a chelating agent, 2- (p-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (SCN-NOTA). Then, ⁶⁸Ga was chelated on the SCN-NOTA-(MNP-SiO₂(NIR797)) by adding ⁶⁸Ga eluted from a ⁶⁸Ge/⁶⁸Ga generator and vortexed at RT for 1 hr. 4 mg/kg of ⁶⁸Ga-(MNP-SiO₂{NIR797}) nanoparticles with 100 Ci of ⁶⁸Ga (291 µg/kg Co+Fe) was subcutaneously injected into the right forepaw of a mouse. The amount of radionuclide was determined by approximately 100

Ci. Figure 7 shows MR image at 1 hour post injection as black spots in the arrow. Some dark spots at the arrow in the MR image represent signals injected by ⁶⁸Ga-(MNP-SiO₂ {NIR797}) nanoparticles. Then, PET image of lymph node was overlaid with CT because PET image does not provide the anatomical information.





Figure 7. (a) MR and (b) PET/CT images of SLNs at 1 hour post injection of ⁶⁸Ga-(MNP-SiO₂(NIR797) nanoparticles subcutaneously into the right forepaw of mouse.

3. Differential Point, Superiority or Characteristics of Technology Applied

Tracking of targeted cell, bio separation and downstream processing are the most important topic among the biotechnology. The materials used so far did not have enough fluorescence and safety to be detected *in vivo*. However, our materials have strong fluorescence in nanoparticles and have not toxicity because magnetic core and organic dyes are coated inside of silica which is recommended as a safe material by experts. The surface of silica is composed of hydroxyl groups and so this functional group can be easily modified with antibodies, proteins and bio-molecules. The specific antibody and protein can play a role as targeting signals to find specific cell or a molecule. These properties can be used for targeting cell to find a disease. Therefore, it is expected that the tracking of targeted cell *in vivo* by optical imaging and early diagnosis of disease are possible.

1. Stability of nanoparticles in vitro and in vivo



Amorphous silica is approved as a safe material by US food and Drug Administration (FDA). Since the surface of our material is silica, it is biocompatible and non-toxic.

2. High efficiency of fluorescence

Fluorescent dyes can be effectively entrapped inside the silica particles and the spectral characteristics of the dye molecules remains almost intact. Silica encapsulation provides a protective layer around dye molecules, reducing oxygen molecule penetration (that causes photodegradation of dye molecules) both in air and in aqueous medium (in this case dissolved oxygen). As a result, photostability of dye molecules increases substantially in comparison to bare dyes in solution.

3. Resistance of photobleaching

Rapid photobleaching is one of the problems with organic fluorescent dyes. Numerous photochemical reactions occur in the cellular environment that can lead to photodegradation of the dye. The encapsulation of the dye in a ceramic matrix is one methodology presently in use to maximize both *in vitro* and *in vivo* stability. This minimizes oxygen access, increases chemical stability and allows surface modification of the shell to enhance hydrophilic character and cell uptake. Different techniques that are in use for encapsulation include incorporation in nucleic acid and PNA oligomers, lipid micelles, polymer matrices and encapsulation in silica matrix.

4. Application

The surface of silica particles can be easily modified to attach bio molecules such as proteins, peptides, antibodies, oligonucleotides, etc., using conventional silane-based chemistry. For example, carboxylated silica nanoparticles can be covalently attached to the amine groups of proteins, antibodies, etc., via the formation of a stable amide bond and peptides containing cysteine residue (via-S-H group) can be attached to the aminated silica nanoparticles.

5. Imaging

Our materials can easily applied for imaging cells in vitro as well as in vivo. The reason is that the intensity of fluorescence is very strong and there is no light scattering in NIR region in vivo. Thus, we can track MSC cells labeled with our materials in vivo without sacrifice for couple of days. This experiment was impossible with Qdot[®] before because Qdot[®] has fundamental toxicity, low intensity and high cost.

Reference

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- 2. H. Youn, K. W. Kang, J.-K. Chung, D. S. Lee, Nucl. Med. Mol. Imaging 2008, 42, 337.
- S. Kim, Y. T. Lim, E. G. Soltesz, A. M. D. Grand, J. Lee, A. Nakayama, J. A. Parker, T. Mihaljevic, R. G. Laurence, D. M. Dor, L. H. Cohn, M. G. Bawendi, J. V. Frangioni, *Nat. Biotech.* 2004, 22, 93.



Specific Patent

NO.	Name of Patent	Application No.	Date of application /approval	Country	Status (Applied/approval)	Cost for patent (KRW)
1	Multifunctional particles providing cellular uptake and magnetic motor effect	10/2005/011 2245	2005.11.23	KR	Approval	
2	Multifunctional particles providing cellular uptake and magnetic motor effect	2006800330 70.0	2008.03.10	CN	Applied	
3	Multifunctional particles providing cellular uptake and magnetic motor effect	2006288048	2008.03.19	AU	Applied	
4	Multifunctional particles providing cellular uptake and magnetic motor effect	2621352	2008.03.04	CA	Applied	
5	Multifunctional particles providing cellular uptake and magnetic motor effect	443/MUMN P/2008	2008.03.10	IN	Applied	
6	Multifunctional particles providing cellular uptake and magnetic motor effect	2008- 529921	2008.03.07	JP	Applied	
7	Multifunctional particles providing cellular uptake and magnetic motor effect	6798700.8	2008.03.31	EP	Applied	
8	Multifunctional particles providing cellular uptake and magnetic motor effect	12/030,848	2008.02.13	US	Applied	
9	Multifunctional particles providing cellular uptake and magnetic motor effect	12/030,863	2008.02.13	US	Applied	
10	Near infrared dye incorporated magnetic core- silica shell multimodal nanoparticles for lymph node and deep tissues optical, MRI and PET image	10/2009/006 5765	2009.07.20	KR	Applied	

* Please provide accurate information for Application No and Date of application/approval. It will be used for patent search.

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In case of PCT or overseas patent (application) except domestic patent, please attach a certificate of application/approval (or patent abstract) as a separate file.