Breast cancer cell targeted MR molecular imaging probe: Anti-MUC1 antibody-based magnetic nanoparticles

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Abstract. Effective and specific diagnostic imaging techniques are important in early-stage breast cancer treatment. The objective of this study was to develop a specific breast cancer contrast agent for magnetic resonance imaging (MRI). In so doing, superparamagnetic iron oxide nanoparticles (SPIONs) were conjugated to C595 monoclonal antibody using EDC chemistry to produce nanoprobe with high relaxivity and narrow size (87.4±0.7 nm). To test the developed nanoprobe in vitro, assessments including Cell toxicity, targeting efficacy, cellular binding, and MR imaging were carried out. The results indicated that after 6 hrs incubation with MCF-7 cells at 200 to 25 µg Fe/ml doses, 76% to 16% T2 reduction was obtained. The presence of iron localised in MCF-7 cells measured by atomic absorption spectroscopy (AAS) was about 9.95±0.09 ppm iron/cell at higher doses of nanoprobe. Moreover, a linear relationship between iron concentration of nontoxic SPION-C595 and T2 relaxation times was observed. This study also revealed that developed nanoprobe might be used as a specific negative contrast agent for detecting breast cancer.

1. Introduction
The MUC1 mucin, a high molecular type transmembrane glycoprotein, is an effective inhibitor for cell-cell and cell-extracellular matrix interactions [1, 2]. Mucin is present at apical surface of epithelial cells and underglycosilated and over-expressed adenocarcinomas cells such as breast cancer [3], ovary cancer [4], prostate cancer [5], and lung cancer [6]. A common characteristic of mucin is a repeated domain which includes (a) an N-terminal extracellular domain, (b) a single membrane spanning domain, and (c) a C-terminal cytoplasmic domain with dense O-linked glycosacharid [7]. Moreover, mucin expression in normal epithelial can be dynamic varying in response to steroid hormone or cytokine influence [8]. Besides, expression of MUC1 on normal tissue is usually at a lower level and characterised by different receptor of immune epitopes. Following malignant transformation, mucin often becomes highly expressed, loses its apical restriction, and displays aberrant glycosylation and altered mRNA splice
variation. In adenocarcinomas, the major change that occurs is the loss of the ordered architecture of the gland, leading to no demarcation of apical and basolateral epithelial cell surface, and as a result mucin is expressed on all around the surface of the cells. As a result, the overexpression of mucin protein generally upregulates where it undergoes changes in glycosylation resulting in the exposure of the core protein tandem repeat region which is a potential target for antibody mediated therapy. Mucin modification in glycosylation and overexpression makes the tumour-associated mucin distinguishable from normal cells [9, 10]. Moreover, it is reported that mucin is expressed in 90% of breast cancers [11]. Generally, the overexpression of mucin and distribution on cell surface are assumed to influence the biological behaviour of the tumour cells during malignant transformation and tumour progression suggesting that protein may be important for maintenance or generation of the tumour. The receptor of immune epitopes to characterise the mucin on malignant cells are BC2(APDTR), HMFG1(PDTR(PA)), HMFG2(DTR), SM3(PDTRP) and C595 (RPAP)[12-16]. Molecular imaging (MI) is one of the non-invasive imaging methods which occurs at cellular and even molecular level. MI also provides information about receptor’s expressions and enzyme activities. The imaging modalities that guarantee MI are PET, SPECT, MRI, and ultrasound. The largest percentage of molecular imaging modalities depend on contrast agents [17]. The recommended agents used for molecular imaging purposes either have a specific goal to bind to the receptors over-expressed at a specific site or are cleaved by enzymes overexpressed at the location of the target.

Cancer, unregulated cell splitting, is one of the most important health issues of the present time and a leading cause of death among populations. Techniques to minimise the morbidity and mortality of this massive health issue include the recognition and modification of the risk factors, early diagnosis and treatment, and improved treatment strategies. EDC (1-ethyl-3-(3Dimethylaminopropyl) carbodiimide hydrochloride) allows for the formation of the direct component as the product of the cross-linkage reacts. An important point of interest in antibodies (Abs) is their antigen-binding region which is extremely specific and different among Abs. The EDC belongs to the groups of carbodiimides or zero-length crosslinking agents. Carbodiimides are widely used for to form amide linkage between carboxylate. In addition, EDC is utilised to interact with biomolecules, by forming an amine-reactive O-acylisourea intermediate. A possible improvement of the present biochemical reaction means that after the cross-linking and amination of the carboxyl coat, NHS esters could be linked to the amine groups on the NP which will react with the amine-reactive O-acylisourea intermediate of the antibody [18-20]. The aim of this study is to determine the efficacy of SPION-C595 as an MR imaging contrast agent targeted MUC1-overexpressing breast cancer under in vitro conditions.

2. Materials and methods

2.1. Chemical
All chemicals were purchased by Sigma (USA). Nanomag-D-spio 20 nm nanoparticles (surface COOH) as well as miniMACS separator in addition to C595 mab were purchased from Miltenyi Biotech GmHb, Germany.

2.2. Cell
MCF-7 breast cancer cells and EA.hy926 normal cells (as control), obtained from ATCC (USA), were routinely cultured in pre-warmed DMEM (37 ºC in water bath) supplemented with 10% of fetal bovine serum (FBS) antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin), 1% v/v essential amino acids, and 2 mM L-glutamine. The cells were grown in culture flasks with media (15 ml media/175 cm²) and incubated at 37 ºC, in a humidified atmosphere of 95%/5% air/CO₂. Also, the cells were harvested after reaching approximately 80% confluence where they were presented as monolayer.

2.3 Conjugation of C595 monoclonal antibody with SPION
Fabrication and the characterisation of the nanoprobe (SPIONs-C595) were performed as described in previous studies [21]. In brief, a 500 µl of the nanomag®-D-spio was mixed with 3 µmol N-ethyl-N-(3-
dimethyl aminopropyl) carbodiimide hydrochloride (EDC) and 10 μmol N- hydroxyl succinimide (NHS) in 125 μl 0.5M 2-(N-morpholino) ethanesulfonic acid (MES) buffer. The suspension was mixed for 90 min at room temperature by shaker. Then, the suspension was washed twice with 1 ml Phosphate Buffered Saline (PBS), pH=7.4 through MS column. After that, a 100 μl MUC1 (C595) mab was added to activate nanoparticles and the suspension was shook for 3 hrs. After 3 hrs the suspension was washed twice with 1 ml PBS through MS column. Then, the 30 μl glycine was added to quench the reaction of the final mixture. Finally, the suspension was passed through the high gradient field for last three times washing with PBS. The amount of immobilised monoclonal antibody (mAb) was calculated by Bradford method.

2.4. Cell viability test
EDC method was utilised to fabricate the nanoprobe. Effect of SPION-C595 on viability of cells was evaluated using MTT assay. Cells from 70-80% confluent cultures were harvested by trypsinization and re-suspended in 5 ml fresh medium. The number of cells was determined and the cell count was adjusted to 5x10^4/ml. Subsequently, 100 μl of cell suspension was seeded in each well and cells were incubated overnight for attachment. The nanoprobe were diluted in cell culture medium to obtain 6 times the final concentration and 100 μl was added to each well. Moreover, the treated cells were incubated for 2 to 48 hrs. Then, a 20 μl of MTT solution was added to the cold cultures medium in each well, and 200 μl of culture medium containing the MTT reagent incubated for 3 to 5 hrs. At the end of the incubation, the supernant was aspirated carefully and the water insoluble formazan salt was solubilised in 100 μl DMSO per well. After 10 min incubation at 37°C, optical density of the violet was measured by a microplate reader at a primary wavelength of 570 nm and a reference wavelength of 620 nm [22].

2.5. Determination of efficacy of nanoprobe
The specific and cellular uptake of C595 functionalised and non-functionalised NPs on MCF-7 cells were examined by Prussian blue staining. Also, cells were seeded at a density of 2x10^5 cells per well. The cells were incubated overnight at 37 °C in a humidified atmosphere of 95%/5% air/CO₂. When the flask was confluent, the monolayer MCF-7 cells were exposed to SPION-C595 and nanomag®-D-spio at 100, and 25 μg Fe/ml of final concentrations for 4 hrs. After the incubation time, the medium of the cells was removed and the cells were washed with PBS and fixed in 4% paraformaldehyde (PFA) for 20 minutes inside an incubator, followed by washing the cells three times. The fixed cells were incubated with 10% potassium ferrocyanide for 5 min and 10% potassium ferrocyanide in 10% hydrochloric acid for 30 min at room temperature (25 °C). The cells were counterstained with nuclear fast red for 5 min after two times washing with PBS. Finally, the fixed cells were washed three times with PBS and were sent for taking image by microscope.

2.6. MR in vitro imaging
For imaging, the MCF-7 cells were grown to confluence in 6-well plates. Different concentration levels (25, 50, 100, 200 μg Fe/ml) of SPION-C595 were prepared in fresh media. Then, breast cancer cells were treated with prepared nanoprobes for 6 hrs incubation time at 37 °C, followed by 3 times washing with PBS, and detaching the cells by the 150 μl trypsin per well. To prepare two million cells for each MR imaging samples, after tyrosination cells were centrifuged and adjusted by counting cell method. The cells were suspended in 250 μl 1% agarose gel in PBS and were transferred into 1.5 ml centrifuge tubes for imaging. Moreover, untreated cells in 1% agarose were considered as negative control (T) while distilled water and (E) 1% agarose were considered as positive controls. The Eppendorf tube box holder was filled with 1% agarose gel to eliminate the background noise. All relaxation times were obtained on a using a 1.5T clinical MRI machine (GE Healthcare, Wisconsin, USA), T₁ and T₂ relaxation time measurements were modifications of the method of previous studies [24, 25]. Furthermore, to determine the slope of the regression line, the relaxivities were calculated by plotting R₁ and R₂ values versus different concentrations of SPION-C595.
3. Results and Discussion

3.1. Cell viability test

As shown in Table 1, SPION-C595 did not exhibit any toxicity toward the cells as the nanoprobe was localized in the cytoplasm. Furthermore, prolonging the incubation of MCF-7 cell with SPION-C595 did not affect either cell morphology or proliferation ability. The results also revealed that there was no toxicity upon conjugation, even at the highest concentration of components (Figure 1). On the other hand, there were statistically significant differences between the concentrations among those within the same incubation time ($P<0.05$ for all) [23, 24].

Table 1. Mean ±SD values of viability percentage of SPION-C595 with MCF-7 after incubation times, among six different concentrations.

| Concentration (µg Fe/ml) | Incubation time | Mean % viability | Total | P-value |
|--------------------------|-----------------|-----------------|-------|---------|
|                          | 2 h             | 8 hrs           | 24 hrs| 48 hrs  |       |
| 1.56 b                   | 106.85 (6.07) c | 82.41 (2.90)    | 154.42 (7.89) | 83.24 (1.15) | 196.73 (30.85) | < 0.001 |
| 3.12                     | 99.59 (2.54)    | 90.20 (3.28)    | 139.57 (17.35) | 77.07 (1.55) | 101.61 (25.54) | < 0.001 |
| 6.25                     | 99.02 (11.81)   | 97.75 (0.96)    | 132.89 (12.45) | 74.70 (0.99) | 101.09 (22.89) | < 0.001 |
| 12.5                     | 109.14 (3.62)   | 106.00 (2.77)   | 141.07 (11.04) | 77.75 (4.37) | 108.49 (24.05) | < 0.001 |
| 25                       | 107.04 (10.39)  | 109.05 (5.40)   | 159.59 (8.71) | 84.84 (3.75) | 115.13 (29.30) | < 0.001 |
| 50                       | 111.71 (5.30)   | 122.43 (1.00)   | 167.82 (6.06) | 68.24 (0.31) | 117.55 (37.15) | < 0.001 |
| Total                    | 105.56 (7.90)   | 101.31 (13.68)  | 149.22 (15.77) | 77.64 (6.03) | 108.43 (28.38) |       |
| P-value                  | NS              | < 0.001         | 0.017 | < 0.001 | < 0.001 |

a Incubation times: after 2 hrs, 8 hrs, 24 hrs, and 48 hrs.
b Concentrations: 1.56, 3.12, 6.25, 12.5, 25, and 50 (µg Fe/ml).
c Arithmetic means [24].

NS: Not significant at 5% level.

3.2. Determination of efficacy of nanoprobe

The z-average hydrodynamic diameter of SPION-C595 was obtained 87.4±0.7 nm with a polydispersity index of 0.3 after conjugation reaction, which was still an ideal size for application of transfection. The
nanoparticle size before conjugation was measured at 51.3± 0.07 nm. The 30 nm increasing in size of nanoparticle revealed that the conjugation occurred [26, 27].

The Prussian blue technique was based on the conversion of ferrocyanide to the insoluble crystal of Prussian blue in the presence of Fe$^{3+}$ under acidic conditions which resulted in the formation of a blue pigment called Prussian blue. The cell was first treated with dilute hydrochloric acid to release ferric ions from the binding proteins. Figure 2 shows the interaction and attachment of nanoprobe to the cancer cells. Attachment to the cytoplasm of the MCF-7 cells due to over expression of MUC1 on MCF-7 cells is shown in blue colour.

The cellular uptake of the SPION-C595 nanoprobe was verified on cancerous (MCF-7) and normal (EA.hy926) cells. Additionally, AAS results showed a 9.95±0.09 ppm iron/cell cellular uptake by MCF-7 cells in comparison to normal cells at higher doses of nanoprobe (Table 2). The quantity of significant (P<0.001) SPION-C595 uptake by MCF-7 confirmed the selectivity of the nanoprobe and its capability for breast cancer detection. Moreover, the results strongly suggested there was no significant cellular uptake by the normal cells in comparison to the breast cancer cells after the administration of a higher SPION-C595 dose.

**Figure 2.** Presence of Fe in MCF-7 cells treated with varying concentration of SPION-C595 nanoprobe. **A** is cells treated with 100 μg Fe/ml of the nanoprobe at 10X magnification. **B** is cells treated with 25 μg Fe/ml of the nanoprobe at 20X magnification. **C** is the negative control untreated MCF-7 cells at 10X magnification. *Note: Fe ions are stained blue, the nucleus of the cells is reddish and the cytoplasm in pink.

**Table 2.** Summary of iron content (Mean ±SD) of EA.hy926 and MCF-7 at different concentration of SPION-C595 after 6 hrs incubation. a Mean value ±SD  b According to two-independent samples nonparametric Mann-Whitney U test. * Significant at level of 0.05.

| Cell line | Concentrations (µg Fe/ml) of iron content of SPION-C595 (ppm iron/cell) |
|-----------|---------------------------------------------------------------|
|           | Control | 25   | 50    | 100   | 200   |
| EA.hy926  | 0.428±0.01a | 0.456±0.03 | 0.505±0.01 | 0.633±0.01 | 0.835±0.02 |
| MCF-7     | 0.030±0.01  | 0.372±0.01 | 0.479±0.01 | 0.954±0.11 | 9.950±0.09  |
| P-value b | < 0.001*    | < 0.001*    | < 0.001*   | < 0.001*   | < 0.001*   |

### 3.3. MR in vitro imaging

Magnetic resonance contrast agents are effective in enhancing MR images by decreasing the $T_1$ and $T_2$ relaxation times through spin-interaction between electron spins of the metal-coating of contrast and the water protons (hydrogen) in tissue. All contrast agents produce changes in magnetic susceptibility by enhancing the local magnetic field. Efficacy of enhancing $T_1$ and $T_2$ weighted images depends on: (a) the time of interaction between the nuclear and paramagnetic substance, (b) the paramagnetic concentration which is directly proportion to the increasing the relaxation time at the lower concentration doses, and (c) the distance between proton nucleus and electronic field. Paramagnetic materials have unpaired electron in the outer orbital shell. By applying magnetic field, the unpaired electrons give rise to magnetic dipoles, and therefore, paramagnetic ions induce large fluctuating magnetic field experienced by nearby protons. If the frequency of fluctuation has a component close to the Larmor
frequency, the significant enhancement of relaxation times will happen. Two mechanism of relaxations are inner-sphere and outer-sphere relaxation. The inner-sphere relaxation occurs between the ions and the water molecules of the tissue, unstable covalent bonds between ions and the water molecules cause chemical interaction of the water molecules and other water molecules in the surrounding environment, and thus enhances the relaxation time. The reduction of $T_1$ will be more obvious depending on the number of bonding. In other word, paramagnetic substance cause greaten transition between spins states. However, the outer-sphere relaxation occurs when the paramagnetic substances greaten the local field which the water molecules are experienced. There is no chemical interaction in this mechanism. The diffusion of the molecules is causing the influence of protons of tissue into the complex. The large heterogeneity of magnetic field around the nanoparticle through which water molecule diffuse, since diffusion induced dephasing of the proton magnetic moment resulting in $T_2$ reduction [28, 29]. In regards to this study, the results demonstrated that only at the 200 μg Fe/ml the signal intensity of the $T_1$ spin-lattice relaxation time decreased by 33% (Figure 3. a and b) [29]. However, the $T_1$ values of the rest of the concentrations were the same as the controls. Also, the percentage of signal intensity reduction ranged from 76% to 16% for the highest to the lowest concentrations. The $T_2$ relaxation time of the SPION-C595s treated cells decreased from 101.1 to 24.81 ms$^{-1}$ in comparison to the $T_2$ relaxation time of the untreated MCF-7 cells at iron concentrations ranging from 25 μg Fe/ml to 200 μg Fe/ml (Figure 3. c and d) [24, 29].

**Figure 3.** The *in vitro* $T_1$-weighted image of MCF-7 cells (a), The graph of $R_1$ versus different concentration of SPION-C595 after 6 h incubation with MCF-7 cells (b), The *in vitro* $T_2$-weighted image of MCF-7 cells (c) and the graph of $R_2$ versus different concentration of SPION-C595 after 6 h incubation with MCF-7 cells (d).

### 4. Conclusion

Early detection of breast cancer is crucial for better diagnosis and chance of survival. For early cancer detection, more accurate, sensitive, and selective disease characterisation is essential when utilising MR imaging techniques. The SPION-C595s exhibited good biocompatibility and safe profile with no significant toxicity was observed which proves good potential clinical applications. A linear dependence was observed between the relaxation rate constants and the iron content of each sample. Thus, the MR *in vitro* images of the breast cancer cells demonstrated that the nanoprobe had potential $T_2$ MR imaging contrast agent for early breast cancer detection.
5. References

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