Preparation and Characterization of Nano-oil bodies for EGFR Overexpression Tumor Cell Targeting

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Abstract

Cancer is one of the most common diseases in the world today. Since 2011, the incidence of lung cancer had exceeded cervical cancer and became the most common female cancer in Taiwan, and the first cause of female cancer death. A novel delivery carrier was developed using artificial oil bodies (AOBs). Plant seed oil bodies (OBs) consist of a triacylglycerol matrix surrounded by a monolayer of phospholipids embedded with the storage protein oleosin (Ole). In this study, a bivalent anti-EGFR affibody domain (ZEGFR2) was fused with Ole at the C terminus. After overproduction in *Escherichia coli*, the fusion protein (Ole-ZEGFR2) was recovered to assemble AOBs. The size of self-assembled AOBs was tailored by varying the oil/Ole-ZEGFR2 ratio and pH to reach a nanoscale. Upon co-incubation with tumor cells, the nanoscale AOBs encapsulated with a hydrophobic fluorescence dye were selectively internalized by EGFR-overexpressing cells and displayed biocompatibility with the cells. As visualized by fluorescent microscopy and confocal microscopy, the Ole-ZEGFR2 was selectively internalized by EGFR positive cells. Moreover, these AOBs were effectively internalized and the fluorescence dye that they carried was subsequently released inside the cells. Taken together, our findings indicate the potential of AOBs as a delivery carrier.

Keywords: nano-oil bodies, EGFR overexpression, lung cancer.

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1. Introduction

Cancer is a leading cause of death worldwide and accounted for 7.6 million deaths (around 13% of all deaths) in 2008 [1]. The main types of cancer is lung cancer. Targeted gene therapy is a subject receiving many intensive studies [2]. As a first attempt, tumor cells which overexpress epidermal growth factor receptor (EGFR) were chosen for targeting. Oil bodies are small spherical vacuoles that appear within the cells of plant seeds, and oil bodies have the size of 0.5-2 μm in diameter on average.

2. Method

Schematic illustration of the protocol for preparing fluorescent Ole-ZEGFR2 AOBs. In the first step, the fusion gene consisting of Ole and the ZEGFR2 motif was constructed and expressed in *E. coli*. Bacterial cells were disrupted and centrifuged to precipitate Ole-ZEGFR2 on the

bottom of the sample tube. Second, the mixture containing recovered Ole-ZEGFR2, oils, and the fluorescent dye was subjected to sonication, resulting in the opaque solution with suspensions. Finally, centrifugation was applied to separate the scum AOBs on the top of the sample tube. Isolated AOBs were dispersed in the solution and visualized by fluorescence microscopy.

3. Results

3.1. Protein production and self-assembly of ZEGFR2-based AOBs

To target EGFR-positive cells, AOBs were modified to display the anti-EGFR affibody. This was done by fusing of Ole with ZEGFR2, and the resulting fusion protein was overexpressed in bacterial strain BL21(DE3)/pJO1-Ole- ZEGFR2.

The control strain BL21(ED3)/pJO1-Ole was cultured in a similar fashion. Self-formation of AOBs was carried out following the general procedure outlined in method. As shown in figure 1(A), the fusion protein was overexpressed in strain BL21(DE3)/pJO1-Ole-ZEGFR2 upon IPTG induction and was mainly present in the insoluble fraction of cell lysate (ppt-1). Ole- ZEGFR2 was then isolated from ppt-1 and employed for self-assembly of AOBs. After centrifugation, AOBs floated on the top of the supernatant (sup-2) and little Ole-ZEGFR2 was left in the cell pellet (ppt-2). Finally, AOBs were subjected to heating to liberate the incorporated proteins. Ole-ZEGFR2 was identified as the main protein. The result indicates the strong association of Ole-ZEGFR2 with plant oil and PLs.

The construction of plasmid pJO1-Ole-ZEGFR2 resulted in the fusion of a 6xHis tag with Ole-ZEGFR2 at the C terminus. As shown in figure 1(B), this fusion protein could be detected with the anti-6xHis tag antibody. Moreover, the anti-6xHis tag antibody was applied and the fluorescence signal was found surrounding Ole-ZEGFR2-based AOBs (figure 1(c)). In contrast, no fluorescence signal could be observed for Ole-based AOBs. Overall, the result suggests the surface display of the fusion partner via Ole onto AOBs.

3.2. Formation of stable nanoscale AOBs

As indicated in table 1, the size of AOBs ranged from 300 to 800 nm, and the zeta -potential of AOBs ranged from -30 to -55 mv. Stability of AOBs was examined by the turbidity test. Figure 2 shows that AOBs made of olive , sesame oil or peanut oil were more stable than those composed of soybean or mineral oil. The result may reflect the distinct TAG constitutions of various oils, leading to various degrees of interaction with ZEGFR2. Accordingly, olive oil was chosen for further experiments.

3.3. Selective internalization of ZEGFR2-displayed AOBs

AOBs were entrapped with a hydrophobic fluorescent dye and assembled in the following conditions: sesame oil/protein ratio of 1:5 and pH 7.5. As a consequence, the size of AOBs ranged from 420 to 500 nm, and their stability remained unaffected when 1 μg ml^{-1} fluorescent dye was encapsulated. Subsequently, the resulting fluorescent AOBs with ZEGFR2 were co-incubated with tumor cells. As shown in figure 3(A), strong fluorescence signals were captured in EGFR overexpressing cells (e.g. A549) whereas the signal was absent in the EGFR negative cells (e.g. HFL-1). The result implies that the ZEGFR2 motif via Ole was correctly presented on the AOB surface, which in turn results in the association of AOBs with

EGFR positive cells. To further confirm the localization of AOBs, *z*-axis scanning fluorescence microscopy and 3D image reconstitution were utilized. The result showed that ZEGFR2-displayed AOBs were located in the cytoplasm of EGFR overexpressing cells (figure 3(B)). Overall, the above-mentioned results imply that ZEGFR2-displayed AOBs were selectively internalized by EGFR positive cells.

3.4. Internalization efficiency of ZH2-displayed AOBs

The internalization efficiency of EGFR positive cells was calculated as the percentage of fluorescence-emitting cells in the entire cell population. First, various doses of AOBs were co-incubated with tumor cells for 2 h. Cells were then collected and processed for analysis by flow cytometry. As shown in figure 4(A), the percentage of cells emitting green fluorescence increased as the dose of AOB increased. This was also confirmed by fluorescence microscopy (figure 4(C)). The maximal efficiency was obtained at MOI 400 AOBs. At that concentration, the efficiency was greater than 90% for EGFR positive cells, A431, and A549.

Tumor cells were also co-incubated with fluorescent AOBs (MOI 200) for various time periods. At the end of incubation, cells were prepared for analysis by flow cytometry. As shown in figure 4(B), the internalization efficiency of ZEGFR2-displayed AOBs correlated positively with the incubation time. A similar observation was obtained with fluorescence microscopy in which the maximal efficiency was reached when the incubation time lasted for 2 h (figure 4(D)).

4. References

[1] Globocan 2008, IARC, 2010. http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900

[2] Suda K, T.K., Mitsudomi T., *Biological and clinical significance of KRAS mutations in lung cancer: an oncogenic driver that contrasts with EGFR mutation.* Cancer Metastasis Rev. 2010 Jan 28.

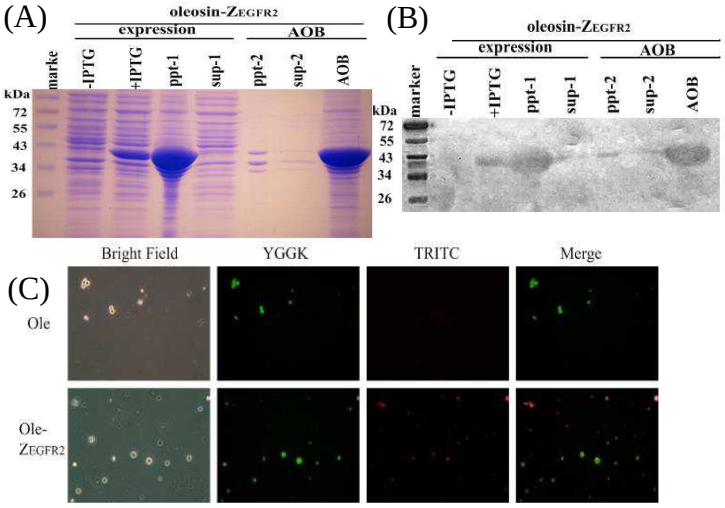

Fig. 1 Analysis of ZEGFR2-displayed AOBs. (A) Recombinant *E.coli* strains were cultured and induced by IPTG for protein production.Toatal proteins from induced (+IPTG) and uninduced (-IPTG) strain BL21(DE3)/pJO1-Ole-ZEGFR2 were resolved in SDS-PAGE. (B) The SDS-PAGE was blotted onto a nitrocellulose membrance and an immunoassay was conducted using the anti-6XHis tag antibody. (C) Encapsulated with a hydrophobic dye (green), AOBs composed of Ole or Ole-ZEGFR2 were against the anti-6XHis tag antibody (red).

Table 1. Particle Size of Ole-ZEGFR2 AOBs prepared at various values of Oil/ protein and pH.

oil/protein Ratio (w/w)	Mean particle size (nm)	pH	Mean particle size (nm)
5:1	394.80±116.30	6.5	488.90±261.80
2:1	487.20±19.40	7.0	542.93±113.82
1:1	780.10±65.30	7.5	508.30±158.57
1:2	805.85±19.95	8.0	540.43±219.25
1:100	550.20±36.00	9.0	622.60±62.01
1:200	680.25±6.850		

Table 2. Zeta-potential of Ole-ZEGFR2 AOBs prepared at various values of Oil/ protein and pH.

oil/protein Ratio (w/w)	Zeta-potential (mV)	pH	Zeta-potential (mV)
5:1	-52.2	6.5	-38.2
2:1	-41.7	7.0	-34.9
1:1	-43.6	7.5	-37.2
1:2	-39.3	8.0	-36.7
1:100	-36.6	9.0	-32.8
1:200	-35.8		

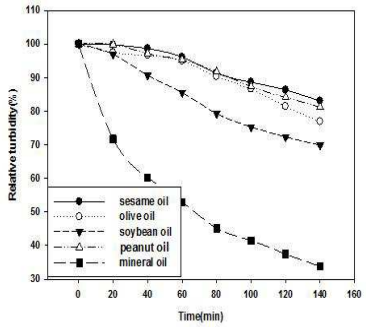

Fig. 2 Stability of Ole-ZEGFR2 AOBs at various conditions. The stability of AOBs was determined by the turbidity test as described. Stability profile of AOBs assembled with various types of oils.

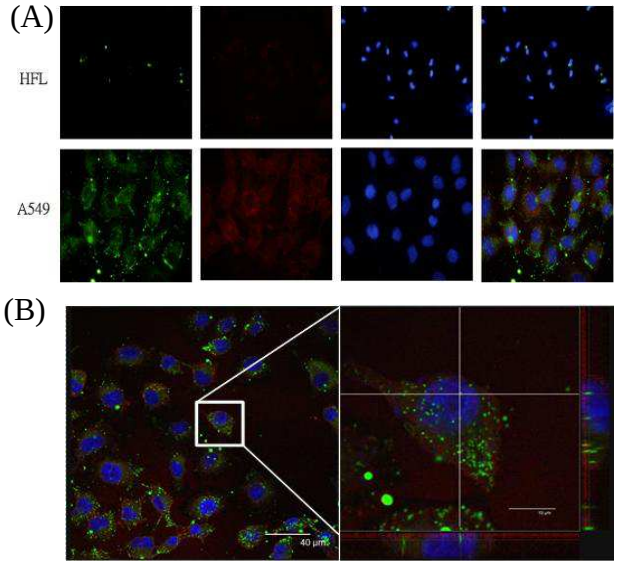

Fig. 3 Selective internalization of AOBs. (A) Analysis by fluorescence microscopy. Cell nuclei (blue) and the EGFR receptor (red) were stained with DAPI and anti-EGFR antibody, respectively. (B) Analysis by confocal laser scanning microscopy.

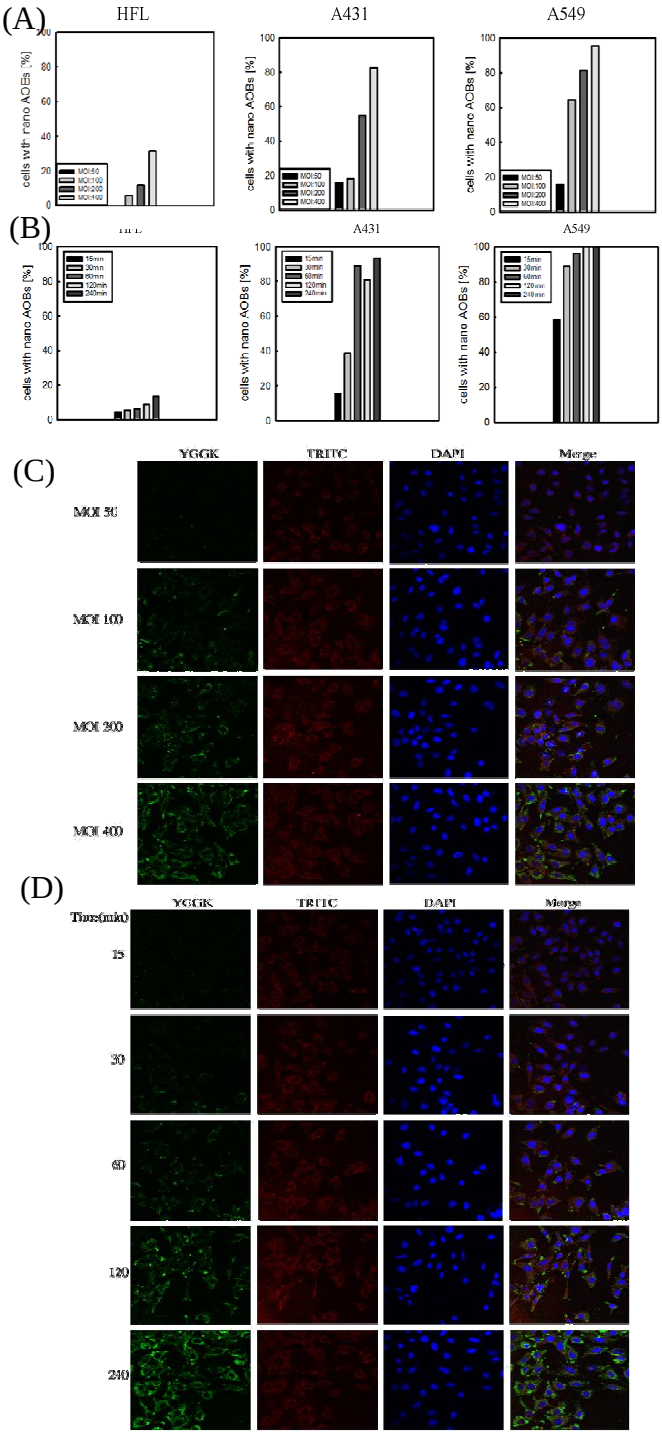

Figure 4. Internalization efficiency of AOBs into tumor cells was assessed by flow cytometry and fluorescence microscopy. (A) Various cells were exposed to Ole-ZEGFR2 of reach the indicated concentration. (B) Various cells were exposed to Ole-ZEGFR2 for an indicated time.(C, D) Tumor cell (A549) treated with various does and various times of Ole-ZEGFR2 AOBs were analyzed by fluorescence microscopy.