Investigation on a fluorescent probe specific to PKM2 dimer and its potential use in isolating cancer-specific exosomes

Abstract
Exosomes are extracellular vesicles that are released upon fusion of the multivesicular bodies with the plasma membrane. They contain various biological materials (proteins, nucleic acids, metabolites, etc.) that represent status of their originating cell. Exosomes are secreted by most cell types in both normal and pathological conditions. The level of tumor-derived exosomes rises significantly in cancer patients. As exosomes are abundant in biofluids such as blood and urine and carry information on originating cells, they can be used for individual and for non-invasive diagnosis. A major challenge in the field is to distinguish tumor-derived exosomes from the sea of normal exosomes. Our lab has synthesized a fluorescent probe that specifically binds to the dimer form of pyruvate kinase M2 (PKM2). PKM2 is a key regulator of cancer metabolism and is one of the proteins commonly identified in exosomes. We would like to investigate whether this probe will be useful in identification and isolation of tumor-derived exosomes.

Introduction
Exosomes: current knowledge on their compositions and diagnostic potentials
Exosomes are a subset of extracellular vesicles that are 40-100 nm in diameter. They are secreted by most cell types in both normal and pathological conditions. They contain various biological materials (proteins, nucleic acids, and metabolites) that are derived from their originating cells [1]. Although initially considered to be involved in cells’ disposal of
superfluous proteins [2], studies have now shown that exosomes are important vehicles for
cell-free intracellular communication. They are implicated in immune response, antigen
presentation, intracellular communication and the transfer of RNA and proteins [1, 2].
Exosomes are derived from multivesicular bodies. Although multivesicular bodies are more
well-known for being a late step in the maturation of endosomes to lysosomes, they could
also take an alternative exocytic fate: their external membrane fuses with the plasma
membrane, resulting in the release of the intraluminal vesicles of the MVBs into the
extracellular environment as exosomes. The exosomes can be internalized by neighboring
cells and facilitate the direct transfer of molecules between cells [3].
Exosomes are secreted by diverse cell types both in vitro and in vivo. They are identified in
body fluids such as urine, amniotic fluid, breast milk, saliva and blood. Proteome studies
have revealed that exosomes contain not only a set of conserved proteins such as tubulin
and kinases, but also cell type/tissue-specific proteins [1]. These cell type/tissue-specific
proteins can be used as biomarkers to identify cell type/tissue-specific exosomes from a
pool exosomes with various origins. This might be particularly useful in diagnosis of cancer.
In cancer patients, the level of tumor-derived exosomes in plasma increases significantly
[1]. Since exosomes contain various biological materials that represent the status of
originating cells, cancer-specific exosomes can be the ideal tool for individual and non-
invasive cancer diagnosis.
While most studies on biomarkers for cancer-specific exosomes focus on surface proteins,
we believe that pyruvate kinase M2 (PKM2), an intracellular protein and a key regulator of
cancer metabolism, is potentially useful in identification of cancer-specific exosomes. PKM2
is one of the proteins that are frequently packaged into exosomes [1]. Although the
inclusion of PKM2 is simply due to exosomes’ ability to engulf cytoplasmic proteins and is not serving any particular purpose [1], it might provide us with a means to identify and isolate cancer specific exosomes.

![Exosome Generation](image)

**Figure 1. Exosome Generation**
The multivesicular bodies (MVBs) can either take on the lytic fate or exocytic fate. Adapted from [3].

2. **Pyruvate kinase M2 (PKM2) plays an important role in cancer cell proliferation**

Pyruvate kinase catalyzes the last reaction in glycolysis: it transfers the high-energy phosphate group from phosphoenolpyruvate (PEP) to ADP, producing ATP and pyruvate. Pyruvate is then either reduced to lactate by lactate dehydrogenase in the cytosol, or directed to the tricarboxylic acid (TCA) cycle in the mitochondria to produce more ATP. While normal cells utilize the more efficient mitochondrial oxidative phosphorylation (TCA cycle) to maximize the production of ATP, cancer cells adopt the less efficient glycolysis even in a well-oxygenized environment. Cancer cells display enhanced glucose uptake and produce a large amount of lactate. This phenomenon is called aerobic glycolysis or Warburg effect. By aerobic glycolysis, cancer cells re-directs more glycolytic intermediates
into biosynthesis of nucleotides, amino acids and lipids, thereby meeting the demands of proliferating cancer cells for cellular building blocks [4].

Pyruvate kinase M2 plays an important role in the altered metabolism in cancer cells.

Pyruvate kinase has four isoforms, and PKM2 is predominant form in cancer cells and other proliferating cells. While other isoforms form stable tetramers, PKM2 exists as both less active dimers and highly active tetramers in cells. Cancer cells express high level of dimeric PKM2. While tetrameric PKM2 favors ATP production through TCA cycle, the less active dimeric PKM2 promote aerobic glycolysis [4,5].

In addition to promoting Warburg effect, PKM2 also promotes cancer cell proliferation through its non-glycolytic functions in the nucleus. Nuclear PKM2 interacts with beta-catenin and contribute to beta-catenin-mediated transactivation of cyclin D and c-Myc. Moreover, nuclear PKM2 acts as protein kinase and phosphorylate histone H3 at T11 in response to EGFR signaling. Collectively, the actions of nuclear PKM2 promote cell proliferation and tumor progression. Interestingly, studies have shown that the functional form of PKM2 in nucleus is the dimer [4,5].

The distinctively high concentration of PKM2 dimers in cancer cells can be utilized to identify cancer-specific exosomes. Since PKM2 is one of the most commonly included intracellular proteins, we would expect cancer-specific exosomes to contain high level of dimeric PKM2 that is not present in exosomes originated from non-cancerous cell types.

3. A cell permeable, fluorescent probe that specifically bind to dimeric PKM2

Since high level of PKM2 dimers are expressed in cancer cells, we think that a fluorescent reagent specific to dimeric PKM2 would be a good candidate for labeling cancer-specific
exosomes. We have generated a series of fluorescent compounds. One of the reagent (binds to human PKM2, and the binding enhances the fluorescence (figure 2), as it creates a hydrophobic environment for the compound, which will increase the fluorescence emission efficiency. Other pyruvate kinase isoforms were not bound to the probe, indicating that this probe is specific to PKM2. We hypothesize that this fluorescent probe will enter the cell and bind with dimeric PKM2. Since PKM2 is usually packaged into exosomes, this probe can potentially be used to label cancer-specific exosomes.

![Figure 2. Fluorescence emission spectra of the probe in the presence of various PKM2 concentration.](image)

**Results**

To test whether the fluorescent probe can go through the plasma membrane and bind with intracellular PKM2, we incubated human cervical cancer HeLa cells in media containing the probe (40 uM) for 24 hours. The cells were then observed under fluorescence microscope (figure 3). It appeared that the probe did enter the cells and stained perinuclear vesicular structures, potentially through binding with PKM2.
Figure 3. Fluorescence microscopic images of Hela cells showing that the fluorescent probe stains some perinuclear structures.

However, with the high concentration of the fluorescent probe (40 uM) we used, we cannot exclude the possibility that the self-aggregation of the probe produced an artificial signal, since aggregation will also create a hydrophobic environment that increases fluorescence efficiency of the probe. To ensure that our observation is not an artifact, we constructed a control probe that is structurally similar to our probe, but lacks the functional group that allows for PKM2 binding (figure 4). The two compounds have similar aggregation tendency. We incubated cells in media containing either the fluorescent probe (40 uM) or the control probe (40 uM) for 24 hours and then observed the cells with fluorescent microscope. We saw fluorescent spots for both conditions, which suggests that most of our signal comes from self-aggregation of the probe in cells, rather than actual binding with the proteins.
To reduce aggregation, we reduced the concentrations of the fluorescent probe. A number of concentrations are tested (0.5 uM, 2 uM, 5 uM, 8 uM, 10 uM and 20 uM), in order to find the lowest concentration required for producing a real signal from protein binding. However, little or no signaling was observed for these lower concentrations.

Finally, to exclude the possibility that some of the probe were actually bound to the PKM2 but were undetected because the bound PKM2 got packaged into secreted exosomes that are washed away in media, we isolated exosomes from culture media with differential centrifugation. We then incubated the exosomes with different concentrations of the probe (0.5 uM, 2 uM, 5 uM, 8 uM, 10 uM and 20 uM). After 1-hour incubation, we washed the samples and obtained emission spectra for both the washes and the samples containing exosomes. For the exosomes-containing samples, we did not see any signal for any concentration of the probe, indicating that the probe is not retained in the exosomes.
Conclusions

To our dismay, the results of our experiments showed that the fluorescent probe is not suitable for labeling exosomes. Although our preliminary data indicates that the probe is able to specifically bind with PKM2 in test tube, it does not work in cultured cells. Low concentrations of the probe is not sufficient to produce any observable signals either inside the cells or in exosomes, while high concentration of the probe tends to self-aggregate and produce false fluorescent signals.
Experimental Procedures

**Fluorescence Imaging**: HeLa cells are maintained in high-glucose (4.5 g/L) DMEM supplemented with 10% FBS. One day before incubation with the probe, we subculture the cells into 96-well plate so that the cells will be approximately 70% confluent the next day. On the day of experiment, we replace the media with media containing the probe and incubate the cells overnight. Cells are then observed under microscope. The fluorescent probe is visualized with DAPI filter. Both fluorescence and phase contrast images are taken.

**Emission Spectra of Exosomes incubated with the probe**: Grow HeLa cells until they are ~ 50 % confluent. Prepare filter sterilized PBS. Collect medium in a 15 mL tube. Record the volume. Detach nearly all cells using trypsin-EDTA. Record the volume of the resuspended cell solution, and count cells. Record the density (cells / mL). Calculate the total cell number. Centrifuge the 15 mL tube with cell culture medium at 2,000 g for 10 min at room temperature. Transfer the sup to 1.5 mL tubes. Centrifuge these tubes at 10,000 g for 30 min at RT. Remove the sup. Resuspend pellet in total 600 uL PBS. Distribute to six different tubes (100 uL each). Add probe or control probe. Incubate at 37°C for 1 hour with agitation. Centrifuge at 10,000g for 30 min at RT. Transfer the sup to new tubes and labeled them as 'Unbound'. Resuspend the pellet in 100 uL PBS, 1% DMSO. Centrifuge at 10,000g for 30 min at RT, and transfer the sup to new tubes labeled as 'Wash 1’. Resuspend the pellet in 100 uL PBS, 1% DMSO. Centrifuge at 10,000g for 30 min at RT, and transfer the sup to new tubes labeled as 'Wash 2’. Resuspend the pellet in 100 uL PBS, 1% DMSO. Centrifuge at 10,000g for 30 min at RT, and transfer the sup to new tubes labeled as 'Wash 3’. Resuspend the pellet in 100 uL PBS, 1% DMSO. labeled as ‘Bound’. Place samples in black 96-well plates and record fluorescence emission spectra.
References:


