On Target

James Song, Lei Zhang and Gang Ren at the University of California, San Francisco investigate the role of individual particle electron tomography as a novel tool for examining the structure of synthetic targeted drug delivery vehicles

Targeted drug delivery has been recognised as a novel approach for delivering medication to specific organs without the need to increase the concentration of the medication (1). In traditional drug delivery systems, such as oral ingestion or intravascular injection, the medication is distributed throughout the body through systemic blood circulation. Consequently, only a small portion of the medication reaches the target organ. However, the ability to increase the concentration of the dose is limited by peripheral toxicity related to the systemic administration of compounds. In contrast, targeted drug delivery is able to concentrate the medication in the tissues of interest without increasing the relative concentration of the medication in the remaining tissues, thus reducing side effects. Targeted drug delivery can be used to treat many diseases, such as cardiovascular diseases, diabetes and cancerous tumours (2). Synthetic targeted drug delivery vehicles, including antibodies, peptides, proteins and vitamins, can enhance the selective uptake of drugs by target tissues or cells (1). However, developing synthetic vehicles can be a cost- and time-intensive process. The ability to monitor the structures of these drug delivery vehicles during and after the manufacturing process could be vital to avoid dead-end development paths and wasted resources.

No technology, so far, can help the drug delivery field to monitor the structure of individual drug delivery vehicles during production, since current technologies, such as x-ray crystallography, nuclear magnetic resonance (NMR) and small-angle scattering, have a common problem that particles need to be relatively homogeneous in order to obtain their structure. However, many vehicles are structurally dynamic and heterogeneous, such as antibodies and lipoproteins. Thus, producing vehicles without monitoring their structures is like taking a stab in the dark. Many vehicle failures are related to structural changes within the vehicles.

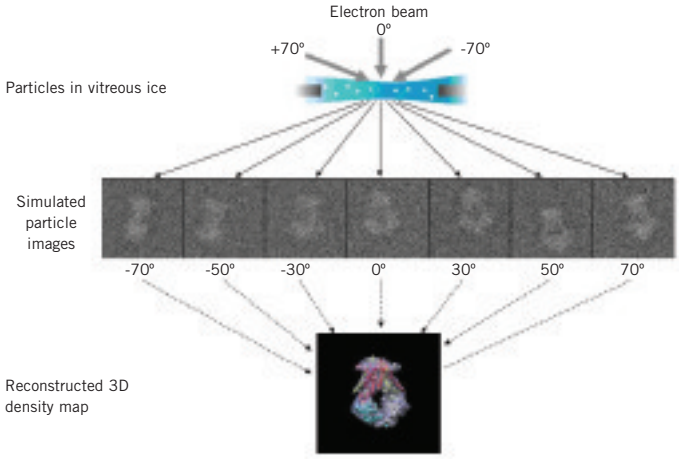
A new method in electron microscopy electron microscopy (EM) called individual particle electron tomography (IPET) overcomes this obstacle by discerning the structure of individual protein particles, allowing for examination of highly heterogeneous, dynamic or flexible proteins. IPET technology allows users to perform a computed tomography (CT) by EM on individual vehicles, and then to build up the three-dimensional (3D) density map of each individual vehicle (3,4) via a focused electron tomographic reconstruction algorithm (5). The map would be critically important for examining the structural changes during the production of vehicles by different production protocols, and for troubleshooting vehicles that lose functionality after modification.

DETERMINATION OF THE DYNAMIC PROTEIN STRUCTURE BY IPET

Protein structure determination by EM has been widely used for decades, the mainstay of which is the single-particle reconstruction method (6). In examining proteins by EM, the particles can either be imaged after negative staining (NS) them, or in their frozen, hydrated state by electron cryo-microscopy (cryoEM). Each method has its pros and cons: negative staining offers good contrast but can cause artifacts or alter protein conformation, while cryoEM allows for native-state observation

Figure 1: A diagram of 3D reconstruction of a dynamic protein by individual particle electron tomography (IPET)

The particles embedded in vitreous ice are tilted and imaged at tilt angles from -70° to +70° in steps of 1°. A total of 141 tilt images are, as an example, generated by simulation of micrographs from cryoEM. After correction of the contrast transfer function (CTF) in the EM images, a 3D density map of the targeted particle is back-projected from the global-aligned particle's images via a local refinement iteration reconstruction algorithm.



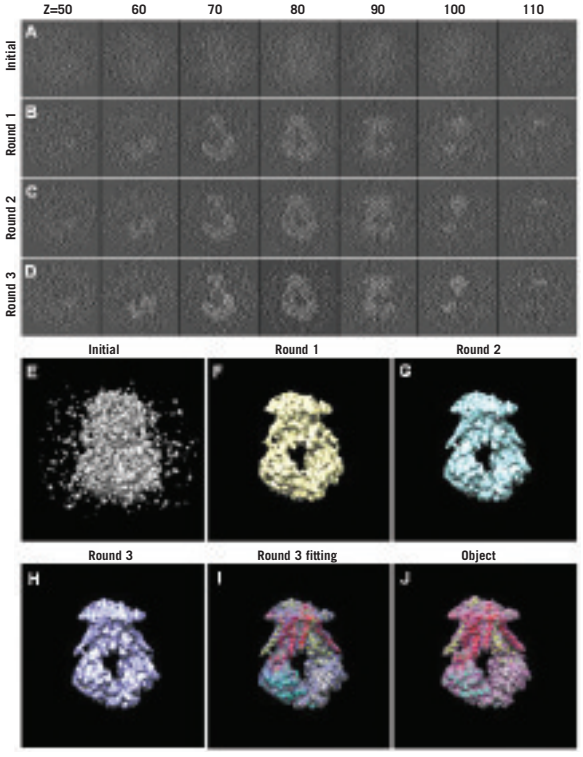
but has low contrast and poor signal-to-noise ratio. Single-particle reconstruction of cryoEM gets around these limitations by grouping similar images and averaging them into 2D classes, which are then used to back-project to a 3D density map (6,7). However, for highly heterogeneous or dynamic proteins such as lipoproteins or antibodies, this averaging can be devastating to fine detail, and can drastically reduce reconstruction resolution.

An IPET method has been proposed as a new approach to resolving the structure of highly dynamic proteins (4). Similar to clinical CT scans, the proteins embedded in vitrified ice are imaged from a series of tilt angles (see Figure 1). By combining the images according to their tilt angles, the software program can reconstruct the 3D density map of each particle.

To achieve high-resolution reconstruction, the particle images need to be aligned precisely. The alignment essentially involves finding the true 3D global centre of gravity in each particle image. The centre searching is performed by an iterative translational searching algorithm that proceeds through three rounds of refinement, each containing multiple iterations (4). In brief, for each iteration, the 3D reconstruction (or an initial model) is generated from the tilt series of particle images and their corresponding tilt angles, then projected to a series of 2D projections. These projections are then compared against the corresponding actual images of the particle to determine the translational parameters in each image. The images are then shifted so that the particle is properly centred in each image and a new 3D map is reconstructed from the re-centred images for the next iteration (see Figure 2). Prior to translational searching, a dynamic Gaussian low-pass filter and circular mask are applied. A low-pass filter is used for removing unnecessary detail and leaving behind the low-frequency information (corresponding to rough shape) needed for centre searching. The boundary frequencies of the low-pass filter – the low-pass frequency and cut-off frequency – are progressively increased to include more high-frequency information as the translational searching converges. This allows the use of progressively finer detail to achieve better alignment. A Gaussian-edged particle-shaped mask is used to eliminate unnecessary background noise. These particle-shaped masks are generated by projecting the running 3D reconstruction by the relevant tilt angle and scaling the projection up in size (so that no truncation of the particle occurs). Initially, a large mask is used. However, as translation searching converges, smaller masks that fit more snugly around the particle image are used. The mask has to be big enough to ensure that no part of the particle is truncated. In the last few iterations of refinement, the translation searching is carried out to sub-pixel accuracy by interpolating the particle images by 10 times in each dimension using the triangular interpolation technique (8). Translation searching is carried out as before, using the smallest mask and finest filter.

Figure 2: A test on simulated cryoEM data shows the high resolution reconstruction details of an individual protein

Selected ET slices of the 3D reconstructions of (A) the initial model, (B) round one, (C) round two, and (D) round three. For comparison, the 3D reconstructions were low-pass filtered to 8 Å, and displayed in iso-surface maps that contain the same volume. The initial model displayed as a globular noisy blob (E), while the reconstructions of the first (F), second (G) and third (H) rounds contained many structural details, such as the α-helices. By docking the crystal structure into the iso-surface from the third round (I), the reconstruction nearly perfectly matched the crystal structure and had no distinguishing differences from the object (J) (4).



The IPET technique has been applied to both simulated data and real images (3,4,9,10). This approach has allowed us to reconstruct the 3D density map of an individual particle at a resolution beyond 10Å (4). To validate the program, a simulated cryoET tilt series based on one particle has been used. The 3D reconstruction of this particle contained enough high-resolution structural details to visualise α-helices (see Figure 2) (4), suggesting this method as a novel approach for high-resolution ET reconstruction that can be used for studying the structure of highly dynamic and heterogeneous proteins.

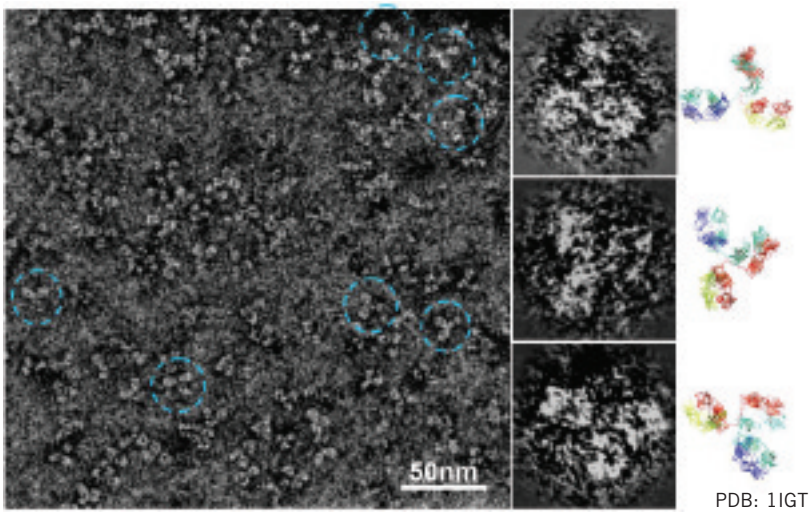
CONJUGATED ANTIBODY STRUCTURE DETERMINED BY IPET

Antibodies have been used as the synthetic targeted drug delivery vehicles that are also frequently used for carrying cytotoxic drugs to kill cancer cells (11). The antibodies respond preferentially to tumour cells, and thus allow for the targeting of tumours. However, during the creation of these drug-carrier conjugates, the binding of the drug to the antibody can cause unwanted conformational change to the antibody (3). This conformational change can reduce the affinity of the antibody to its antigen, and can reduce the effectiveness of the drug or render it useless. For instance, CovX Research LLC developed a peptide that had promise as a potent and selective drug candidate, but did not succeed primarily because of poor pharmacokinetics. The fusion of the peptide to a scaffold antibody produced a conjugated antibody molecule, CovX-Body. The conjugated antibody protects the peptide from renal elimination and enzymatic degradation (12). However, it is possible that the fusion process may itself affect the intrinsic properties of the antibody scaffold (such as effector function).

Changes in function may relate to changes in structure. Thus, we studied the structures of conjugated and unconjugated antibodies with high-resolution EM (see Figure 3) and IPET (see Figure 4) (4). Hundreds of high-resolution images and 3D density maps of individual conjugated and unconjugated antibodies demonstrated the detailed structures of the antibodies, including holes (less than 10Å in diameter) in the Fab domains (see Figures 3 and 4). Comparing the high resolution images and reconstructed 3D maps demonstrated that:

Figure 3: High resolution EM images of unconjugated antibodies demonstrate the structural details of domains

Selected antibodies (circles) on the high resolution EM micrographs (left panel) show three domains in each antibody. Three selected antibody particles (middle panel) show each domain contains a hole (less than 10 Å in diameter) that matches the crystal structure of antibody (PDB: 1IGT) (4).



- The average angle between the Fab regions was 55° ± 15° in unconjugated antibodies, but 40° ± 10° in conjugated antibodies
- The average sizes of the Fc domain were similar regardless of conjugation
- The Fc domain shape differed significantly depending on conjugation

The Fc domains of conjugated antibodies were significantly elongated (more than 30 per cent) after fusing with the drug, suggesting that the elongation of the Fc domain may cause reduced affinity of the antibody to its antigen. This example demonstrates that IPET could be a useful tool for monitoring the structural changes of drug delivery vehicles.

MODEL OF HUMAN LOW DENSITY LIPOPROTEIN AND BOUND RECEPTOR BASED ON CRYO-EM

Another class of drug delivery vehicle is the lipoprotein, particularly low-density lipoprotein (LDL) and high-density-lipoprotein (HDL) (13). LDL is the major carrier of cholesterol in human plasma for cell growth →

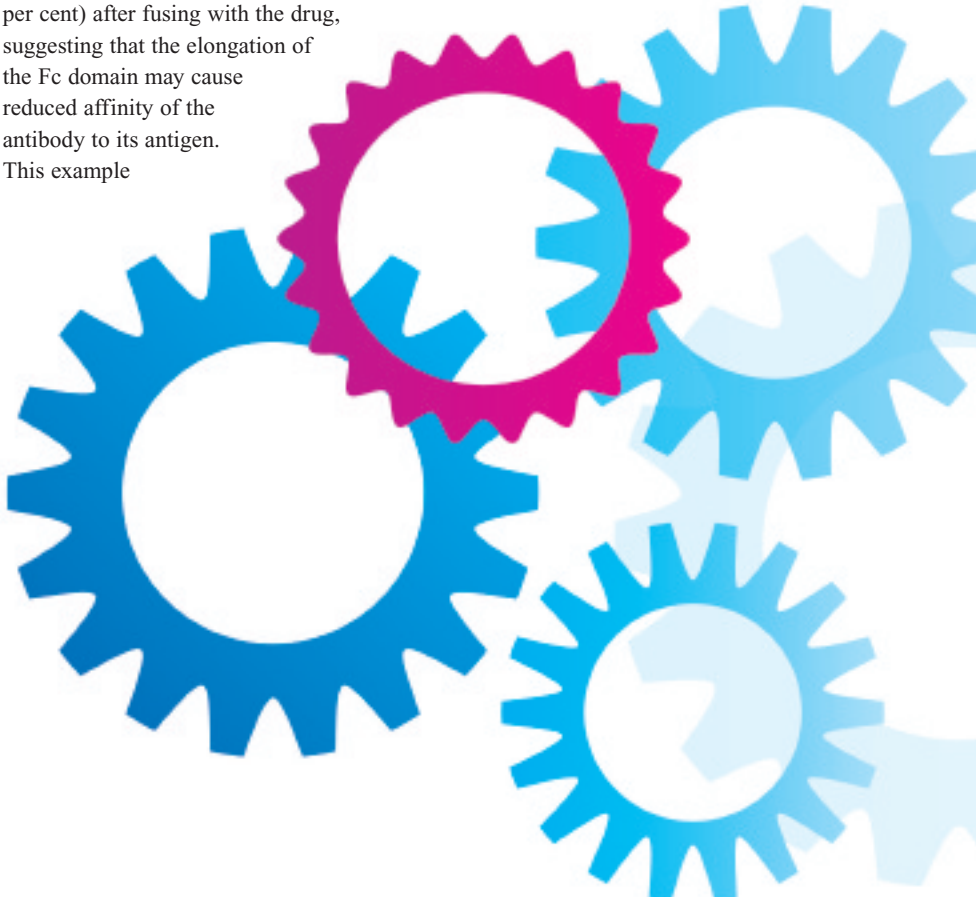
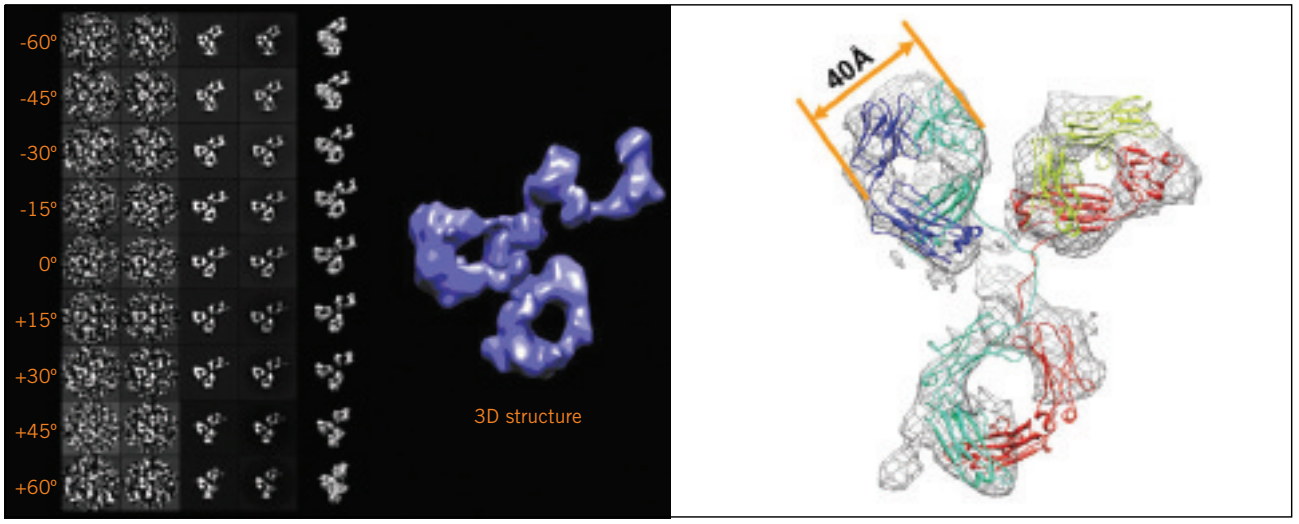


Figure 4: A 3D density map of an individual unconjugated antibody as reconstructed by IPET

Views from micrographs taken at the specified tilt angles are shown in left panel. The last three columns show the results of progressive refinement by the IPET algorithm. Reconstruction of an individual antibody particle demonstrates the detailed structure of three domains (middle panel), in which the angles between the Fa, Fb and Fc domains were measured and compared between conjugated and unconjugated antibodies. Density maps of each domain can be reasonably well fitted with corresponding domain structure truncated from the crystal structure of the antibody (PDB: 1IGT) (4).



and membrane repair. Lipophilic drugs can reside safely within the hydrophobic core of the lipoprotein. In lipid metabolism, LDL is removed from plasma by hepatic LDL receptors (LDLr) (14,15) that bind to apoB-100, a 4,536 amino acid polypeptide component of LDL. The LDL is then endocytosed and degraded in a lysosome, causing the drug to be released. Tumour cells, such as in hormone unresponsive breast, malignant brain, prostate, adrenal and lung tumours have an abnormally high level of LDL receptors, as they generally have high cholesterol requirements to support rapid cell division (16-18). Thus, LDL is an ideal drug delivery vehicle for treating tumours.

Researchers have demonstrated, through *in vitro* studies, that synthetic LDL particles can be used as a safe and effective means of delivering anticancer drugs to glioblastoma multiforme tumours (19). The synthetic LDL particles consist of commercial lipids and a synthetic peptide, the LDLr binding domain of apoB (19). This synthetic LDL is useful because plasma LDL is difficult to isolate in large quantities due to its variability in composition and size, and the apoB-100 protein is difficult to isolate due to its large size and propensity to aggregate. The manufacturing process of the synthetic peptide can be modified such that the peptide will be recognised by other receptors on other cells.

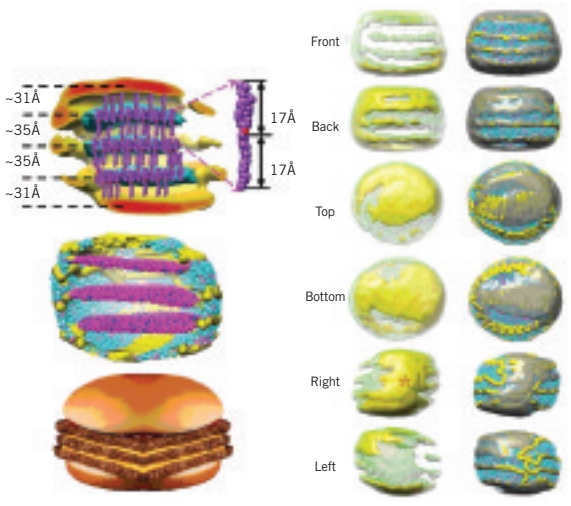
Since the structure of the lipoprotein plays an important role in targeting and delivery, the structure of LDL would be very important for designing drug vehicles. Thus, we determined the structures of LDL and its complex with the LDL receptor extracellular domain (LDL•LDLr) at extracellular pH by single-particle reconstruction of cryoEM (15). Difference imaging between LDL•LDLr and LDL localises the site of LDLr bound to its ligand. The structural features revealed from the cryo-EM map lead to a juxtaposed stacking model of cholesteryl esters (CEs) (see Figure 5). High density in the outer shell identifies protein-rich regions that can be accounted for by apoB-100, leading to a model for the distribution of α-helix and β-sheet rich domains across the protein surface (see Figure 5). The structural relationship between the apoB-100 and CEs appears to dictate the structural stability and function of normal LDL. The normal LDL structure may help us to understand the function difference by comparing the structure of normal LDL with that of synthetic LDL revealed by IPET.

CONCLUSION

IPET provides a simple and elegant solution for the high-resolution structure determination of protein drug carriers such as antibodies. This technology gets around the limitations of other techniques that require that the protein to be determined be relatively rigid and homogeneous. The ability to determine the structure of these drug carriers can be very useful in their analysis and

Figure 5: Structural model of LDL revealed by cryoEM

High density regions of LDL reconstructed 3D density map are shown in the middle panel (yellow and green). This allowed us to map the pentapartite model of apoB-100 to the high density region of LDL map, consisting of two amphipathic β-sheet rich domains interrupted by two amphipathic α-helix-rich domains (shown in yellow cable). The internal structure of LDL resembles a triple cheeseburger, with juxtaposed regions of protein and cholesteryl esters (left panel), the phospholipid head groups, CE and TG are displayed as cyan, magenta and blue balls respectively (15).



design, as conformational changes during the production process that would render the drug ineffective can be identified without having to waste a whole clinical trial. This could potentially lower costs and improve the efficacy of drugs and the efficiency of their development.

References

1. Torchilin VP, Drug targeting, *Eur J Pharm Sci* 11 Suppl 2: ppS81-91, 2000
2. Sellers WR and Fisher DE, Apoptosis and cancer drug targeting, *J Clin Invest* 104(12): pp1,655-1,661, 1999
3. Zhang L, Kaspar A *et al*, Monitoring the structural changes of CovX-bodies by high-resolution electron microscopy and individual-particle electron tomography, 54th Biophysical Society Annual Meeting, San Francisco, *Biophysics Journal*, 2010
4. Zhang L and Ren G, Determining the dynamic protein structure by individual-particle electron tomography: an individual antibody structure at a nanometer resolution/ Structural determination of heterogeneous protein by individual-particle electron tomography – combination of electron tomography and local refinement reconstruction method for high-resolution structural determination of each individual protein particle, 54th Biophysical Society Annual Meeting, San Francisco, *Biophysics Journal*, 2010
5. Zhang L, Song J *et al*, An optimized negative-staining protocol of electron microscopy for ApoE4.POPClipoprotein, *J Lipid Res*, 2009
6. Ludtke SJ, Baldwin PR *et al*, EMAN: semiautomated software for high-resolution single-particle reconstructions, *J Struct Biol* 128(1): pp82-97, 1999
7. Frank J, Radermacher M *et al*, SPIDER and WEB: processing and visualization of images in 3D electron microscopy and related fields, *J Struct Biol* 116(1): pp190-9, 1996
8. Isaac A, Scattered data interpolation methods for electronic imaging systems: a survey, *Journal of Electronic Imaging* 11(2): pp157-176, 2002
9. Zhang L, Cavigiolio G *et al*, Structure of 9.6nm discoidal high-density lipoprotein revealed by individual-particle electron tomography, 54th Biophysical Society Annual Meeting, San Francisco, *Biophysics Journal*, 2010
10. Zhang L, Cavigiolio G *et al*, A method to study the time-dependent remodeling of high density lipoproteins by individual-particle electron cryo-tomography, 54th Biophysical Society Annual Meeting, San Francisco, *Biophysics Journal*, 2010
11. Oldham RK, Monoclonal antibodies in cancer therapy, *J Clin Oncol* 1(9): pp582-90, 1983
12. Doppalapudi VR, Tryder N *et al*, Chemically programmed antibodies: endothelin receptor targeting CovX-Bodies, *Bioorg Med Chem Lett* 17(2): pp501-506, 2007
13. Masquelier M, Vitols S *et al*, Low-density lipoprotein as a carrier of antitumoral drugs: *in vivo* fate of drug-human low-density lipoprotein complexes in mice, *Cancer Res* 46(8): pp3,842-3,847, 1986
14. Mahley RW and Innerarity TL, Lipoprotein receptors and cholesterol homeostasis, *Biochim Biophys Acta* 737(2): pp197-222, 1983
15. Ren G, Rudenko G *et al*, Model of human low density lipoprotein and bound receptor based on Cryo-EM, *Proc Natl Acad Sci USA*, 2009
16. Nakagawa T, Ueyama Y *et al*, Marked hypocholesterolemia in a case with adrenal adenoma-enhanced catabolism of low density lipoprotein (LDL) via the LDL receptors of tumor cells, *J Clin Endocrinol Metab* 80(1): pp92-96, 1995
17. Stranzl A, Schmidt H *et al*, Low-density lipoprotein receptor mRNA in human breast cancer cells: influence by PKC modulators, *Breast Cancer Res Treat* 42(3): pp195-205, 1997
18. Chen Y and Hughes-Fulford M, Human prostate cancer cells lack feedback regulation of low-density lipoprotein receptor and its regulator, SREBP2, *Int J Cancer* 91(1): pp41-45, 2001
19. Nikanjam M, Gibbs AR *et al*, Synthetic nano-LDL with paclitaxel oleate as a targeted drug delivery vehicle for glioblastoma multiforme, *J Control Release* 124(3): pp163-171, 2007

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