Molecular Recognition of Complementary Liposomes in Modeling Cell – Cell Recognition

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KEYWORDS:

cell recognition · liposomes · membranes · molecular recognition · phospholipids

1. Introduction

Liposomes due to their structural features are considered as the closest analogues of biological cells. They can therefore be used as models for simulating cell – cell interactions or interactions of cells with molecules of the extracellular environment. It is well established[1] that some cell interactions are transient such as those between cells of the immune system and the interactions that direct white blood cells to sites of tissue inflammation. In other cases, stable cell – cell junctions play the key role in the organization of cells in tissues.[1, 2] It will therefore be challenging to elaborate, through molecular engineering processes, the shape, stability, and recognizable properties of the external surface of liposomes to render these particles susceptible to interaction with simple molecules or other liposomes. In this manner their behavior may mimic rather closely the processes occurring at the external surface of cells. Analogous phenomena may also be encountered in the interaction of living cells with liposomes when the latter are employed as drug delivery systems.[3, 4]

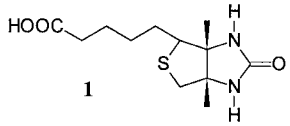
In connection with the recognition effectiveness of the interacting groups, as determined by their specific molecular structure, molecular recognition is enhanced relative to isotropic media since the interacting groups are located at the organized liposomal interface.[5–7] In fact, the binding constants between complementary moieties differ by some orders of magnitude for various types of molecular organized aggregates.[5]

Cell – cell adhesion realized by the interaction of the glycocalyx carbohydrate coat with proteins such as selectins or integrins[1] was simulated by designing mixed liposomes. Such experiments were performed[8, 9] and reviewed[10, 11] years ago involving interactions followed by agglutination of polymerized liposomes, prepared from amphiphilic carbohydrates, with lectins, specifically concanavalin A. On the other hand, quite recently an artificial carbohydrate-binding receptor based on phospholipids bearing a boronic acid moiety has been employed[12] since it has been established that boronic acid derivatives act as synthetic saccharide-binding receptors.[13, 14] Carbohydrate – protein recognition has thoroughly been investigated[2] justifying a separate review by itself.

In this Minireview we discuss some examples of molecular engineering the interacting surface of liposomes through the incorporation, among others, of recognizable moieties. The biomaterials obtained, characterized as tissue-like composites, were investigated primarily by microscopic and spectroscopic studies. Interacting liposomes were prepared following the strategy of incorporating recognizable moieties at the interface of liposomes and allowing the latter to interact either with simple molecules in the aqueous environment, or with other complementary liposomes. However, since liposomal adhesion and fusion has also been achieved electrostatically or, in some cases, by combined electrostatic forces and hydrogen bonding, some typical examples will be briefly discussed before proceeding to liposome aggregation that is mediated exclusively by hydrogen bonding based on molecular complementarity. Finally, proceeding one step further towards the biological reality, liposome – cell and cell – cell interactions will be discussed, employing recognizable moieties analogous to those used in the liposomal systems.

2. Interaction of liposomes with molecules dissolved in aqueous environment

The recognition of biotin (**1**) by streptavidin (see Figure 1 A) is associated with an exceptionally high binding constant of



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$10^{15}\,M^{-1}$, and it has been extensively investigated,[15, 16] being one of the first systems employed for assembling liposomes. Specifically, Chirovolu et al.[17] prepared mixed unilamellar liposomes that were based on dilauroylphosphatidylcholine and contained dipalmitoylphosphatidylethanolamine-conjugated biotin as a recognizable molecule. When streptavidin was added to the biotinylated liposomal dispersion aggregation occurred, followed by precipitation.

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Cryoelectron microscopy of the resulting liposomal aggregates showed that only minimal changes in their shape and size occurred. Tethered liposomes were formed through biotin–streptavidin bridges[18] as shown schematically in Figure 1B. Most of the liposomes were found in large aggregates, while only few free liposomes were seen in the micrographs. The aggregates were bound strongly, withstanding breakage even under the relatively large shear forces exerted during sample preparation for cryoelectron microscopy.

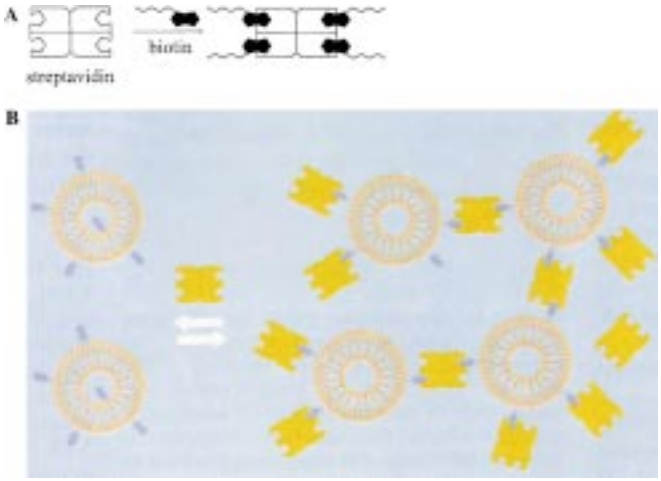
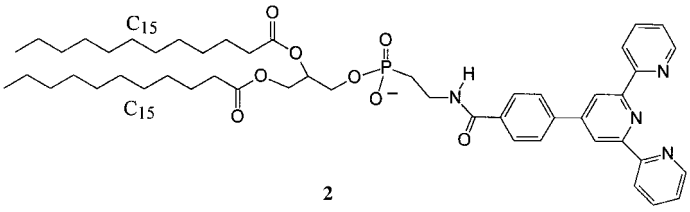


Figure 1. *A: Interaction of biotin with the tetrameric protein streptavidin. B: Schematic representation of the aggregation of biotinylated liposomes induced by interactions with streptavidin. (Reproduced from ref. [18] with permission.)*

The analogous association of mixed liposomes that were based on lecithin and contained the terpy-functionalized phospholipid **2** (terpy = 2,2′,2″-terpyridine) was induced by the



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presence of Fe^{II} ions. Addition of the metal-complexing agent disodium ethylenediaminetetraacetate (Na_2edta) reversed the association process, leading to the formation of a simple liposomal dispersion[19] as shown schematically in Figure 2. This process, however, cannot be generalized since—depending on the interacting species—even destruction of the original liposomes may occur. This is the case with liposomes interacting with the guanidinium cation ($C(NH_2)_3^+$) and its derivatives.[20] On titrating a dispersion of dihexadecylphosphate liposomes with an aqueous solution containing guanidinium cation or its derivatives, including arginine, the liposome size decreases and they are finally transformed to micelles at a 1:1 molar concentration ratio of the interacting species. In this case, the guanidinium counterions are bound to the phosphate groups by combined electrostatic and hydrogen-bonding forces and since

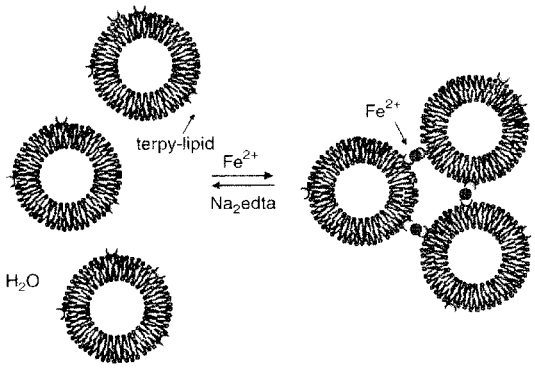


Figure 2. *Aggregation of terpy-functionalized liposomes induced by iron(II) ions and dispersion of the aggregates into simple liposomes after the addition of Na_2edta. (Reproduced from ref. [19] with permission.)*

their sizes are such that the surfactant parameter requirement[21] [Eq. (1)] is fulfilled, micelles are formed.

$$\frac{V}{\alpha l} < {}^1/_3 \qquad (1)$$

In this formula V is the volume per hydrocarbon chain, or of the hydrophobic region of the surfactant, l is an optimal hydrocarbon chain length related to the maximum extended length, and α is the head group area.

3. Interliposomal interactions through electrostatic forces or hydrogen bonding

3.1. Interactions between oppositely charged liposomes

Incorporation of positively or negatively charged lipids, at appropriate concentrations, in liposomes originating primarily from neutral lipids, induces their interaction. Two possible contact interactions can occur, that is, *adhesion*, in which the liposomes conjoin, but retain separate inner compartments; and *fusion*, in which the particles merge sharing a common inner compartment. These phenomena and the elucidation of the respective mechanisms have been studied systematically employing primarily spectroscopic methods for assessing the dynamic features of adhesion or fusion together with electron microscopy. Lately, studying giant liposomes[22] and employing video-enhanced optical microscopy it became possible to observe these liposomal interactions in real time.

In some early experiments,[23] mixed liposomes based on phosphatidylethanolamine and phosphatidylcholine and containing low molar fractions of either a cationic lipid, 1,2-bis(oleyloxy)-3-(trimethylammonium)propane, or an anionic lipid, phosphatidylserine, were allowed to interact. The main conclusions of these investigations, as derived from detailed turbidity and fluorescence studies, may be summarized as follows: The composition of neutral lipid liposomes affects significantly both their initial contact and also the subsequent interactions. These further interactions between liposomes, which can induce mixing of their lipids and aqueous interiors, are surprisingly promoted rather than hindered by increasing ionic strength. It was also shown that cationic liposomes rich in

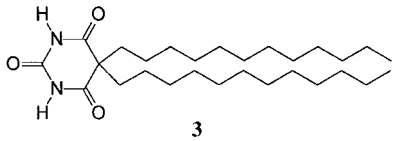
phosphatidylethanolamine, a lipid with relatively weak surface hydration, can assemble and intermix with natural membranes or liposomes predominantly consisting of phosphatidylcholine, which usually resist the processes of bilayer association and coalescence.

In an analogous study, Lehn et al.[24] studied the interactions between charged unilamellar liposomes of various sizes that were prepared from phosphatidylcholine and cholesterol also containing charged components originating from dihexadecylphosphate and octadecylamine. Detailed characterization with ^{133}Cs NMR spectroscopy, light scattering, and electron and optical microscopy showed that oppositely charged liposomes interact through contact followed by lipid exchange. The progressive charge neutralization as shown by ^{133}Cs NMR occurs without fusion of the internal pools of liposomes. Liposome size is of crucial significance since it determines contact duration, extent of lipid exchange, and distribution of surface charge among the liposomal mixture.

In this connection giant charged liposomes were recently prepared[22] consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and cholesterol, and containing 1 – 20 mol % cationic or anionic lipids. Depending on the concentration of ionic components these liposomes of opposite charge either adhere slowly on contact or adhere with severe membrane distortions and even membrane layering. Microscopic observations also showed that bursting of a cationic liposome attached to an anionic liposome could be stimulated by adhesion of a second cationic liposome to the anionic liposome at a site distant from the first contact. As already mentioned these processes were followed by video-enhanced microscopy in real time.

3.2. Interactions between liposomes bearing complementary moieties at their surface

Taking another step towards the elaboration of liposomal surface in order to make it susceptible for specific interactions, complementary moieties having the capability of recognizing each other through hydrogen bonding were introduced at their external surface. For this purpose complementary amphiphilic 5,5-didodecylbarbituric acid (DBA, **3**) or 9-hexadecyladenine (HDA, **4**) were incorporated at approximately $^1/_7$ molar ratio to



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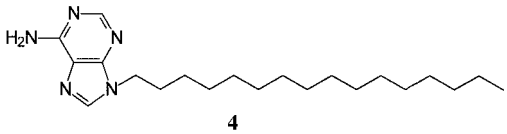

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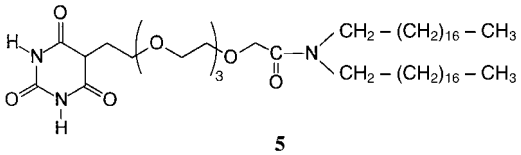
didodecyldimethylammonium bromide (DDAB) leading to the formation of mixed liposomes.[25] Phase-contrast optical microscopy and atomic force microscopy (AFM) have shown that interaction of these mixed liposomes led to the formation of larger aggregates. This interaction and fusion to bigger aggregates, following the collision of the mixed liposomes, can only be attributed to the recognition of their complementary moieties, located at the external interface of the liposomes, since both particles are positively charged and, therefore, they would normally repel each other. It is interesting to note that the mixed liposomes consisting of DDAB and either DBA or HAD exhibit excellent stability: to destroy them, more than about 40 % (v/v) ethanol has to be added. It is obvious that the incorporation of amphiphilic barbituric acid and adenine derivatives results in the stabilization of the simple DDAB liposomes in a way reminiscent of the stabilization of the liposomes by the incorporation of cholesterol.

In analogous experiments larger liposomes were also obtained by the interaction of a complementary pair of liposomes, prepared from lecithin and containing amphiphilic derivatives of barbituric acid and triaminopyrimidine (**5** and **6**, respectively).[26]



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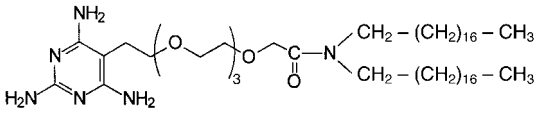

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The recognition of the complementary moieties was facilitated by the insertion of a suitable spacer in between the hydrophilic and lipophilic groups. Freeze-fracture electron microscopy was employed for following the aggregation process of the mixed liposomes as shown in Figure 3.

Molecular recognition of liposomes, induced by biotin – streptavidin recognition as described above, was used amongst others in producing multicompartmental aggregates of tethered liposomes encapsulated within a large liposome. These aggregates (called vesosomes)[27] were prepared by an elegant but rather tedious procedure. Large aggregates of unilamellar liposomes were prepared[17] employing biotin – streptavidin tethers, the size of which was reduced through extrusion, producing in this manner a dispersion of compactly sized liposomes with diameters ranging from 0.3 to 1.0 μm. These aggregates were encapsulated within an outer bilayer by interaction with cochleate cylinders, which are biotin-functionalized multilamellar lipid tubules formed spontaneously by certain negative phospholipids, such as phosphatidylserine, in the presence of Ca^{II} ions. The formation is schematically shown in Figure 4. The formation of vesosomes does not only establish the applicability of molecular recognition for the construction of elaborate biomimetic structures, but it also provides an elegant method for the preparation of vehicles for multifunctional or multicomponent drug delivery systems.

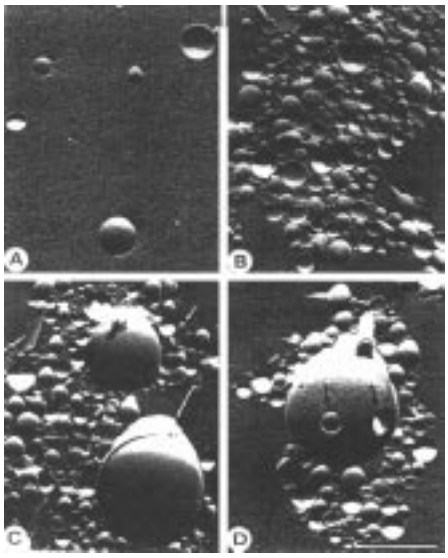


Figure 3. *Freeze-fracture electron micrographs of mixed liposomes composed of lecithin and lipids **5** or **6** before and after mixing. A: Image of lecithin liposomes containing lipid **5** or **6** before mixing. B: Aggregation immediately following mixing of the complementary liposomes. Images C and D were obtained after incubation for at least 15 minutes. (Reproduced from ref. [26] with permission.)*

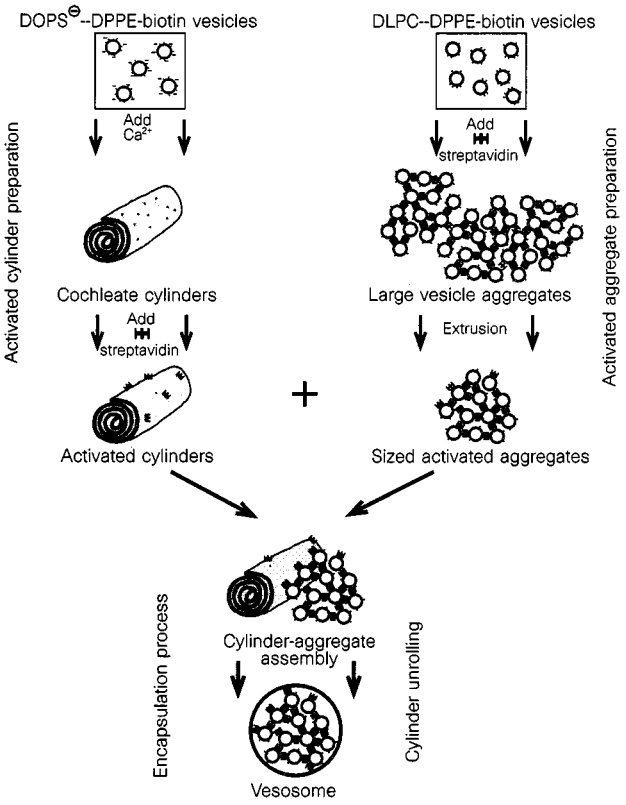


Figure 4. *Schematic representation of the process for the preparation of vesosomes. (Reproduced from ref. [27] with permission.) DLPC = dilauroylphosphatidylcholine, DOPS = 1,2-dioleoylphosphatidylserine, DPPE = dipalmitoylphosphatidylethanolamine.*

Cholesterol, being a basic constituent of cell membranes, is known to affect liposome structure and properties and therefore experiments with liposomes incorporating varying amounts of cholesterol may be simulating the function of cholesterol in cell – cell recognition. For this purpose, a pair of liposomes was used,[28] originating from hydrogenated phosphatidylcholine (PC) and incorporating dihexadecylphosphate (DHP) and 1-(4-(dihexadecylcarbamoyl)butylguanidinium *p*-toluenesulfonate (DBG), respectively. Guanidinium and phosphate groups interact strongly due to the combined action of electrostatic forces and hydrogen bonding.[5]

These complementary liposomes interact spontaneously upon mixing and the resulting liposomal aggregates were observed with phase-contrast optical microscopy. The initially formed aggregates, due to unreacted recognizable moieties, interact further resulting in even larger aggregates, which in certain cases encapsulate smaller liposomes as shown in Figure 5. This behavior is reminiscent of the results of previous

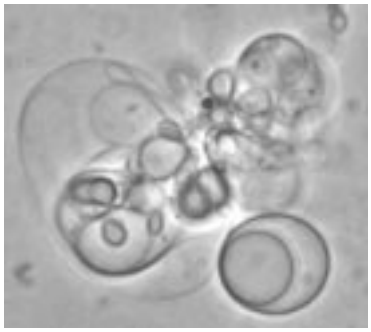


Figure 5. *Phase-contrast optical microscopy images of liposome aggregates immediately following mixing of the samples.*

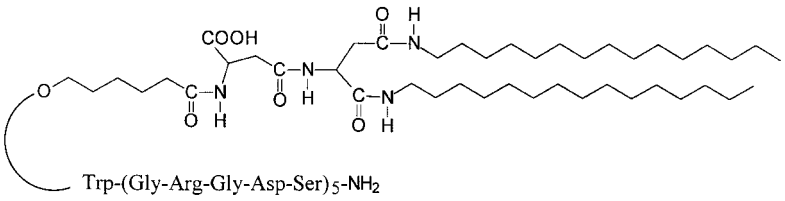
experiments[27] obtained during the recognition of liposomal aggregates with cochleate cylinders, which led to the encapsulation of liposomal aggregates. As concluded[28] from turbidimetric measurements and isothermal titration calorimetry, cholesterol incorporated in liposomes significantly enhances their molecular recognition effectiveness. It is thus possible to tune the association capability of liposomes by changing the incorporated amounts of cholesterol in the liposomal bilayer. Based on this, model liposomal systems may be produced in order to explore the role of cholesterol in molecular recognition in cell membranes and drug delivery targeting.

4. Liposome – cell and cell – cell interactions through complementary moieties located at the external surface

Proceeding to systems simulating closer the behavior of biological cells, liposome – cell and cell – cell systems were investigated employing synthetically prepared recognizable molecules, which were incorporated in the liposome bilayer. A detailed work by Papahadjopoulos et al.[29] set the framework for these interactions. Thus, employing two mammalian cell lines, CV1 and J774, and using fluorometry these authors reached the following conclusions: Liposome uptake was dependent both on the surface properties of the liposomes and on the cell lines. Thus, negatively charged phospholipids incorporated into lip-

osomes that were formed from phosphatidylcholine and cholesterol (2:1 molar ratio) were recognized by the two cell lines to a different extent. This is dependent on the lipid headgroups and their charge density in the liposome bilayer. Thus, inclusion of 9 mol% phosphatidylserine (PS), phosphatidylglycerol (PG), or phosphatidic acid (PA) promoted the uptake of liposomes by CV1 cells more than 20-fold. Increasing the percentage of these negatively charged lipids beyond 9 mol% did not further increase the uptake. Also, inclusion of 9 mol% PS, PG, PA, or PI (phosphatidylinositol) in phosphatidylcholine/cholesterol liposomes did not enhance the uptake by J774 cells, but a drastic enhancement was observed when increased concentrations of these ionic lipids were incorporated into the liposome bilayer. The final conclusion is that the rate of liposome uptake is not only controlled by the negative charge on the liposome surface but also, as expected and desired, by the specificity of the head group of the lipid. Furthermore, higher surface charge density also promotes uptake, but the concentration of the negatively charged lipids required for high-level uptake is dependent on the cell type.

In an analogous manner the lipopeptide RGD-C4A2 (**7**) was anchored with its lipophilic moieties in phospatidylcholine liposomes prepared by the well-established film method. These surface-modified liposomes were found to bind to the membrane of NIH3H3l cells through intermolecular interactions.[30]



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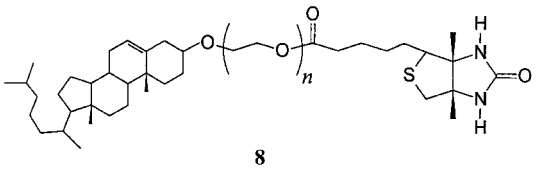
Making another step towards the real world of biological systems the aggregation of cells was achieved by employing synthetic, properly functionalized poly(ethyleneoxides) (PEOs, **8**).[31] Specifically, unsymmetrically substituted PEOs bearing at one end a hydrophobic cholesteryl group and at the other end a hydrophilic biotin group were anchored in the membrane of SubT1 cells (a human CD4-expressing T-lymphoblastoid cell line) through their cholesteryl group following incubation for 4 h at 37 °C. On the other hand, the length of the PEO spacer must be such as to protrude out of the glycocalyx of the cell to be accessible for interaction with the added streptavidin. Therefore, the molecular weight of the PEO spacer chain was varied between 2000 and 35 000 g mol^{-1}, that is, its root-mean-square end-to-end distance being approximately 6 to 20 nm. The interaction between biotinylated cell membranes and streptavidin is



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shown schematically in Figure 6 together with optical microscopy images of the cells. It is interesting to note that aggregation is reversed by addition of free biotin to the cell dispersion in a large excess. It is therefore reasonable to assume that the biotinylated polymer has a significantly lower binding affinity for streptavidin as compared to that of free biotin. As claimed by the authors, this streptavidin-mediated aggregation is not only limited to lymphoblastoid cells; it can also occur with hepatocytes or bacteria.

5. Concluding remarks

The functionalization of liposomes at their external surface has proved an effective strategy for inducing their assembly. Functional groups were introduced at the stage of liposome formation employing various lipids including the recognizable (e.g. biotin-labeled) ones by well-established methods. In cells, the incorporation of functional moieties was achieved by their incubation in the presence of molecules bearing a lipophilic membrane-anchoring group. Complementarity of the functional groups and a favorable interplay[32] of entropic and enthalpic effects is the driving force for the formation of these aggregation biomaterials that have, in certain cases, multicompartmental properties. Although research in this field is still far from its ultimate goal, that is, constructing tissue-like structures, the first steps have already been made and intensification of the effort together with ingenious experiments are required for accomplishing this goal. It is hoped that this Minireview will stimulate interest in this kind of research that is instrumental for organogenesis.

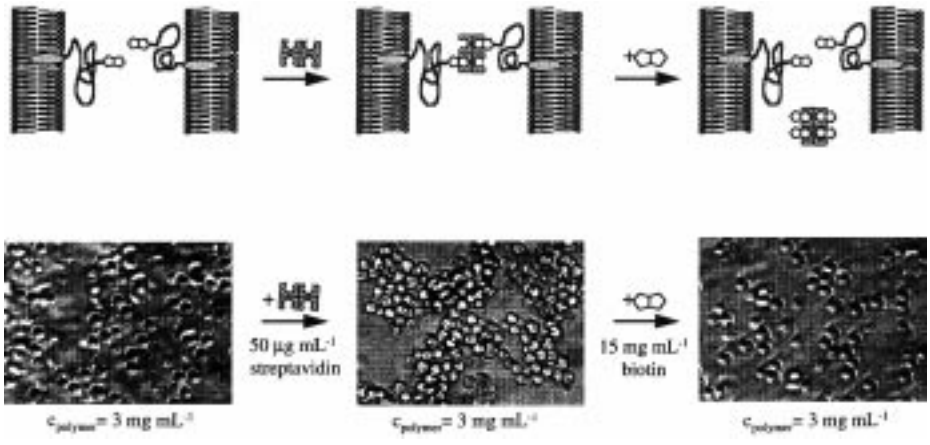

Figure 6. *Top: Schematic representation of bridging biotinylated cell membranes through binding to streptavidin. Bottom: Photomicrographs of the same process using SubT1 cells. (Reproduced from ref. [31] with permission.)*

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