Vesicle and Cell Networks: Interconnecting Cells by Synthetic Polymers

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Water-soluble polymers carrying a small fraction of hydrophobic groups are able to insert these groups into the hydrophobic interior of lipid bilayers or membranes. Under certain conditions such polymers can interconnect different vesicles or biological cells. With increasing polymer concentration this leads to the formation of a three-dimensional network. In this paper we report about the preparation and a first characterization of the structure and the mechanical properties of these new vesicle−polymer and polymer−cell gels composed of α,ω-cholesterol-modified poly(oxyethylene) and dimethyldioctadecylammonium chloride vesicles or living cells.

Introduction

Vesicles or liposomes are spherically closed lipid bilayers with an aqueous interior. They can be regarded as simple models for biological cells or cell membranes and, therfore, have become important for biochemical and biophysical investigations. Their interactions with polymers can be utilized to modify the bilayer surface (e.g. to stabilize these model membranes by a synthetic polymer scaffold) and the colloidal properties of vesicle suspensions.[1] While vesicles usually aggregate and fuse due to depletion forces in the presence of hydrophilic polymers,[2−4] their interactions with polymers carrying a low fraction of hydrophobic groups are of greater interest. These so-called hydrophobically modified water-soluble polymers can be anchored in the membrane via the hydrophobic moieties (e.g. alkyl groups or lipids).[1] The driving force is the gain of energy associated with the transfer of the hydrophobic groups from a partially solvent-exposed state in a micellar system to the more ordered hydrophobic environment in the membrane. This surface-grafting of water-soluble polymers leads to steric stabilization even in a biologic milieu, a necessary condition for their utility as drug carriers for anticancer therapy and targeted drug delivery.[5]

It is obvious that polymers bearing more than one anchor group should be able to bridge two vesicles by inserting their anchors into the vesicular membranes.[6] Certain conditions, however, must be fulfilled: one expects an equilibrium between loop (polymers which fold back to the same membrane surface) and bridge (polymers interconnecting different aggregates) forming polymers. This equilibrium sensitively depends on the mean distance of the hydrophobic groups along the polymer chain and the average vesicle−vesicle distance in the solution (given by the vesicle concentration).[7,8] Only when both are compatible will a significant fraction of bridging polymers be formed.

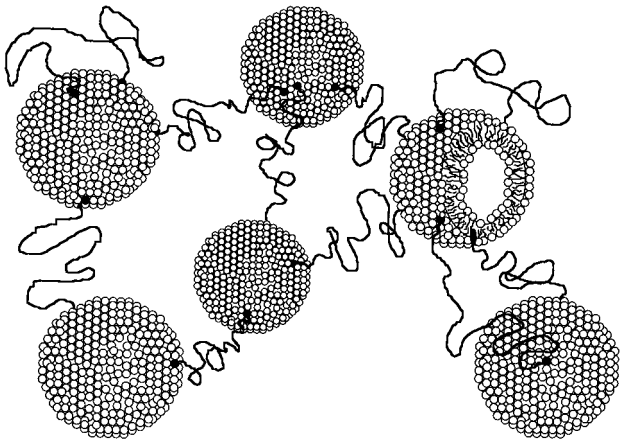

Figure 1. Schematic representation of a vesicle−polymer network.

As a consequence, in a vesicle suspension of given concentration, increasing polymer concentration leads to an increasing number of bridges and finally to the formation of a system-spanning three-dimensional polymer network (see Figure 1). The cross-links are constituted from the individual vesicles together with the hydrophobic groups of the polymers. Due to the dynamics of the lipid molecules in the membrane, the cross-links of the network structure have a finite lifetime; i.e., these systems can be described as transient networks[9,10] with interesting dynamic and rheologic properties.[11]

Regarding the vesicles as simple models of biological cells, these supramolecular networks propose an intriguing mechanism for artificial cell colony formation: incorporating their end groups into biological membrane polymer bridges may also interconnect intact cells.

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Polymers containing two hydrophobic groups with a defined distance along the polymer chain are for example α,ω-hydrophobically modified poly(oxyethylene)s, i.e. poly(oxyethylene)s bearing alkyl groups at each end of the polymer chain. In view of the significance of cholesterol for the structure and stability of biological membranes, cholesterol end groups seem to be well suited as anchor units of the polymer.[12]

In this work we report about polymer gels formed by solutions of cholesterol end group modified poly(oxyethylene) in vesicle suspensions. These new materials are characterized by cryo-electron microscopy and rheological investigations. The first experiments to use this polymer network formation to induce agglomeration of living biological cells are described.

Experimental Section

Materials. Dimethyldioctadecylammonium chloride (DODAC) was twice recrystallized from acetone, subsequently freeze dried from benzene to remove water traces, and stored in a desiccator between the individual experiments. Our experiments require rather large quantities of lipid. We used therefore DODAC, which is a cheap lipid component and is accessible at a high degree of purity. Water was doubly distilled.

The vesicles were prepared using ultrasonication in water at a concentration of 2×10^{-2} mol L^{-1} and subsequent extrusion through 0.1 μm Millipore filters ($T = 60$ °C).

α,ω-Cholesterol-modified poly(oxyethylene) was prepared from poly(oxyethylene) with a molecular weight of 35 000 g mol^{-1} ($M_w/M_n = 1.08$, using GPC) and cholesteryl chloroformiate following the procedure of ref 13. The degree of substitution was found to be larger than 95% by ^{1}H-NMR.[13]

The polymer was dissolved in the vesicle suspension and stirred until the mixture appeared homogeneous. It has to be emphasized that in contrast to poly(oxyethylene) the end-capped polymer is not completely miscible with pure water. In the diluted concentration regime (which is of interest in the context of our experiments) phase separation occurred into a low-viscosity water phase and a gel-like polymer-rich phase. This is probably due to the large hydrophobic cholesterol moieties, leading to a very low critical aggregation concentration for the end groups.

Assuming Gaussian statistics for the polymer coil and excluded volume interaction, the end-to-end distance of the poly(oxyethylene) is calculated to be 29 nm.[14]

The overlap concentration c^* of the polymer chains in solution can be estimated from $c^* \approx M/N_A R_G^3$,[15] with N_A Avogadro's number and R_G the radius of gyration. We obtain $c^* \approx 37$ g L^{-1}, which is significantly higher than the highest polymer concentrations used in our experiments (20 g L^{-1}); i.e., the concentration of the polymer remains dilute.

SupT1 cells (a human CD4 expressing T-lymphoblastic cell line) were grown in suspension in RPMI medium supplemented with 10% FCS (fetal calf serum), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg of streptomycin. The number of cells in the samples (about 2×10^6 cells per milliliter of suspension) was always approximately the same. For the experiment the SupT1 cells were washed with phosphate-buffered saline (PBS), centrifuged, and subsequently taken up in a polymer solution (in RPMI medium) of the respective concentration. After addition of the polymer the different cell suspensions were gently mixed, plated on 35 mm plastic tissue culture dishes, and incubated for about 18 h at 37 °C.

Measurements. Kinematic and (via the density) steady-state shear viscosity data were obtained with an automatic Ubbelohde viscosimeter, Vicometic MS (Fica, France) equipped with thermostated capillaries. The absence of a remarkable shear rate dependence of the viscosity was confirmed qualitatively by measurements with different capillary diameters and by using a PAAR-modified falling ball microviscosimeter, allowing continuous variation of the shear rate. Samples with higher polymer concentrations showed a slight shear rate dependence of viscosity, which was negligible compared to the polymer concentration dependence.

Dynamic mechanic investigations were performed using a Carrimed CRSH 100 controlled stress rheometer combined with a laboratory made cone−cone geometry and a thermostable cap (±0.05 K). For a detailed description see refs 16 and 17.

Freeze−Fracture Replication Transmission Electron Microscopy. About 10 μL of the sample material was brought onto a gold platelet at room temperature and was quenched by hand plunging into a mixture of 2-methylbutane and propane (about 15% 2-methylbutane) at 83 K. After quenching the sample was transferred into liquid nitrogen and clamped on a brass block (Balzer). It was mounted on a Balzer freeze edge device (BAF300), and subsequently the pressure was reduced to 5×10^{-7} mbar. After evacuation the sample was fractured with a liquid nitrogen cooled microtome. To enhance the contrast of the surface structure, the sample was warmed up to 153 K and edged for 10 min. Thereafter the sample was cooled again with liquid nitrogen and shadowed with Pt/C under an angle of 30°. After the samples were warmed up to room temperature and brought to atmospheric pressure, the replica was washed with chloroform and put on a 400 mesh copper TEM-grid and examined with a Hitachi H-8000 electron microscope operating at 100 kV.

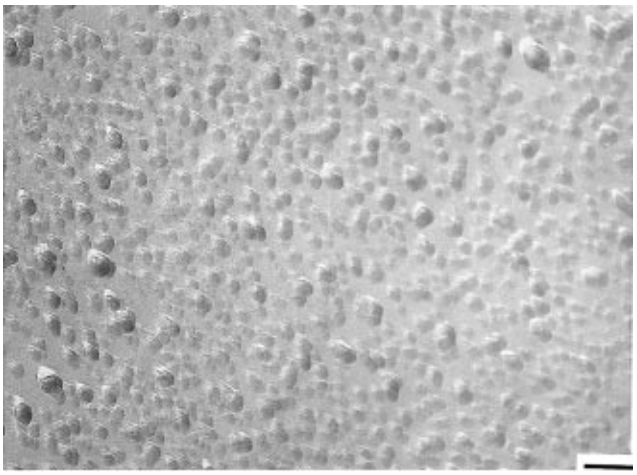

Figure 2. Freeze−fracture electron micrograph of a polymer-containing vesicle suspension (2×10^{-2} mol L^{-1} DODAC, $c_p = 5$ g L^{-1}, six months after preparation). The length of the bar corresponds to 200 nm.

Results and Discussion

The hydrophobic compartments in the lipid bilayer of a vesicle or liposome offer a good "solvent" for the cholesterol end groups of the modified polymer. As a consequence—in contrast to the case in pure water—the functionalized polymers are very soluble in vesicle or liposome suspensions. Figure 2 shows a micrograph of a polymer-containing vesicle suspension. The figure indicates that the vesicle structure is preserved in the presence of the polymer. The average vesicle diameter and center-to-center distance can be directly measured from the micrograph, yielding 90 ± 20 and 110 ± 20 nm, respectively. The errors are mainly due to the polydispersity of the vesicles, to a restricted visibility of the vesicles, being partially buried after the fracture, and to difficulties to distinguish them from their surroundings. The corresponding average distance from vesicle surface to vesicle surface is about 20 nm, i.e. smaller than the root-mean-square end-to-end distance of the polymer (about 29 nm).

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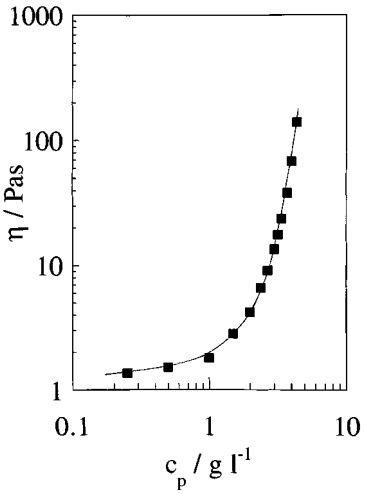

Figure 3. Variation of the viscosity of a vesicle suspension (2 × 10^{-2} mol L^{-1} DODAC) with the concentration of the α,ω-cholesterol-modified poly(oxyethylene) (T = 298 K).

Consequently, a fraction of the polymers is always able to bridge different vesicles (by inserting the cholesterol ends into the lipid bilayer) without changing their natural end-to-end distance, i.e., without loss of entropy.

As a result the formation of a vesicle/polymer network with increasing polymer concentration is observed, which is for example directly reflected by a dramatic increase of the viscosity of the system already at rather low polymer concentration (see Figure 3). It has to be emphasized that for corresponding unmodified pure poly(oxyethylene) no comparable viscosity effect was observed within the concentration regime regarded. Moreover, while the mixtures containing the cholesterol-modified polymers were homogenous, transparent, and stable over a period of more than six months, samples containing pure poly(oxyethylene) appeared turbid after a couple of days. This difference is probably the result of a steric stabilization due to the surface-grafted cholesterol-modified polymer and a depletion force induced vesicle aggregation and fusion in the presence of the pure water-soluble polymer.[2−5] Preliminary experiments with other lipid components (POPC (1-palmitoyl-2-oleyl-3-*sn*-phosphatidylcholine), lecithin) show qualitatively the same behavior.[18]

In order to evaluate the mechanical properties of these new vesicle−polymer gels, rheological measurements were performed. Typical plots of the storage moduli $G'(\omega)$ for systems with constant vesicle and varying polymer concentrations c_p are shown in Figure 4a. Samples with $c_p \geq 5$ g L^{-1} display only a plateau typical for polymer networks in the experimentally accessible frequency range. Typical for gelating systems above the gel point, the frequency dependence of $G'(\omega)$ decreases with increasing polymer concentration.[19]

Due to the finite lifetime of the cholesterol groups in the lipid bilayer (i.e. the cross-links of the network structure), however, the samples are always able to flow on long time scales. The relative narrow frequency range, which prevents measurements at lower frequencies relevant in this context, had to be chosen because of the limited sample stability due to solvent evaporation in the rheometer (see refs 13 and 16). To determine the typical time scales involved with viscous flow of these transient networks, creep experiments under improved conditions will be performed.[18]

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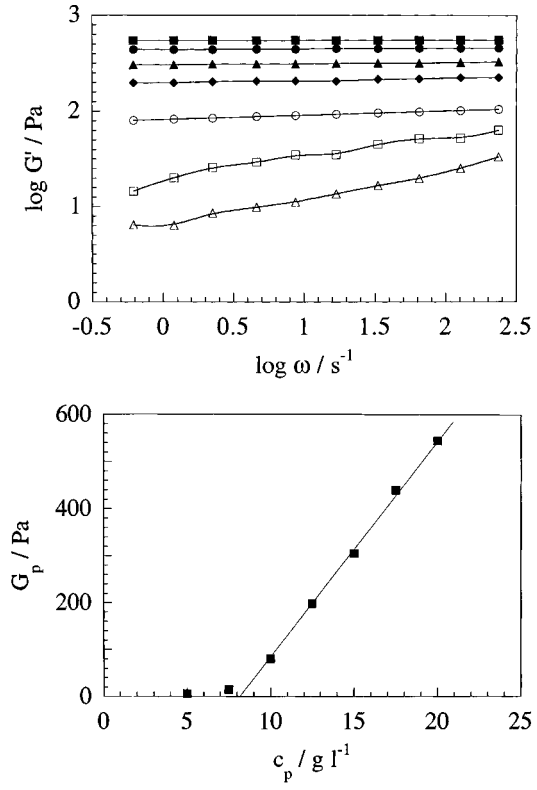

Figure 4. (a) Storage moduli G' of vesicle suspensions (2 × 10^{-2} mol L^{-1} DODAC) with different polymer concentrations, c_p (T = 293 K): (■) 20.0 g L^{-1}; (●) 17.5 g L^{-1}; (▲) 15.0 g L^{-1}; (◆) 12.5 g L^{-1}; (○) 10.0 g L^{-1}; (□) 7.5 g L^{-1}; (△) 5.0 g L^{-1}. (b) Plateau moduli, G_p, plotted against polymer concentration, c_p (2 × 10^{-2} mol L^{-1} DODAC).

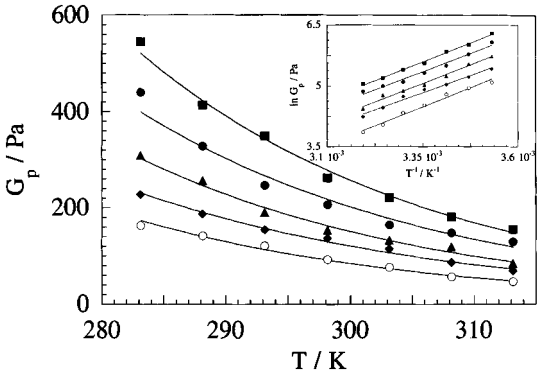

Figure 5. Temperature dependence of the plateau moduli, G_p, for vesicle suspensions (2 × 10^{-2} mol L^{-1} DODAC) with different polymer concentrations, c_p: (■) 20.0 g L^{-1}; (●) 17.5 g L^{-1}; (▲) 15.0 g L^{-1}; (◆) 12.5 g L^{-1}; (○) 10.0 g L^{-1}. Inset shows plot of ln G_p against T^{-1}.

As shown in Figure 4b the plateau value G_p of $G'(\omega)$ grows linearly with c_p at higher polymer concentrations. This observation supports our picture of a network structure formed by polymer and vesicles together. Therefore, the number of cross-links of the network structure is given by the number of vesicles in the system and consequently is constant throughout the whole series. Increasing polymer concentration increases only the functionality and not the number of cross-links. Coincident with our result, theoretical considerations predict for this case $G_p \propto c_p$.[20]

The temperature dependence of G_p is shown in Figure 5. The plateau modulus decreases by a factor of three in the temperature interval regarded. Since only the bridge-forming fraction of the polymer contributes to G_p, lower values of G_p directly reflect a decreasing number of polymer

chains bridging different vesicles ($G_p \propto$ number of bridging polymers according to classical rubber theory[20,21]).

As already mentioned above, the number of polymer molecules which are able to interconnect different vesicles depends sensitively on the average vesicle−vesicle distance and the end-to-end distance of the polymer. The freeze−fracture replication of Figure 3 indicates that the distance from vesicle surface to vesicle surface is always smaller and the vesicle diameter larger than the end-to-end distance (i.e. $\approx$ 29 nm). As a consequence the formation of both bridges and loops is possible without loss of entropy for the polymer chains. That means in equilibrium equal fractions of bridges f_b and loops f_l are to be expected; i.e., $f_b/f_l = 1$. In the temperature regime regarded, the vesicle−vesicle distance and the end-to-end distance of the polymer (water is always a good solvent for poly(oxyethylene) at $T \leq 40$ °C) do not alter significantly:[22,23] the ratio of bridges to loops remains constant at $f_b/f_l = 1$. Nevertheless the temperature dependence of G_p indicates a considerable decrease of the number of bridging polymers which must be the result of the increasing formation of energetically less favored species like dangling ends (with one cholesterol end group exposed to water). As a consequence of the miscibility gap in the diluted polymer/pure water system the fraction of free chains (both ends exposed to water) is expected to be negligibly small. Then the temperature dependence of G_p must be governed by the energy difference between bridges and dangling ends, i.e., polymer chains with both hydrophobic moieties inserted into a lipid bilayer of a vesicle and those having one cholesterol end in direct contact with water. The ratio of the fraction of dangling ends f_d to the fraction of bridges f_b is given by

$$\frac{f_d}{f_b} = \text{constant} \exp\left(-\frac{\Delta\epsilon}{kT}\right) \quad (1)$$

with $\Delta\epsilon$ being the transfer energy for one cholesterol group from a bilayer into water.[7] As shown in the inset of Figure 5, a plot of ln G_p ($\propto$ ln f_b!) against T^{-1} for different polymer concentrations (eq 1) is able to describe well the experimental results. The slope of the straight line is found to be independent of the polymer concentration and yields $\Delta\epsilon = 30 \pm 2$ kJ mol^{-1}. This agrees reasonably well with the literature data ($\Delta\epsilon \approx 40$ kJ mol^{-1}) for the transfer energy of cholesterol from water into a micelle or bilayer.[24]

Due to the use of vesicles and liposomes as simple models of biological cells, it is tempting to investigate also the influence of the α,ω-cholesterol-modified poly(oxyethylene) on living cells. The main question is if the polymer molecules are also able to interconnect different cells, thus leading to a network structure comparable to that of the vesicle−polymer gels.

In our model system the vesicles, the vesicle−vesicle distance, and the end-to-end distance of the polymer are roughly of the same dimensions. In contrast to this, living cells usually have a size and average cell−cell distances in the micrometer region, i.e., exceed by far the dimensions of the polymer molecules. Additionally the cell membranes are enveloped on their outside by the glycocalix formed by oligosaccharide groups of glycoproteins and glycolipids. Both eventually hinder the bridging process, since bridging of two cells by the α,ω-cholesterol-modified polymer can only occur if the two cells approach each other in the nutritive solution to about 30 nm, i.e., the mean end-to-end distance of the polymer. The formation of one bridge, however, prevents a subsequent separation of the two cells. The presence of several polymers in the intercell region may lead to a cooperative reinforcement of the cell−cell

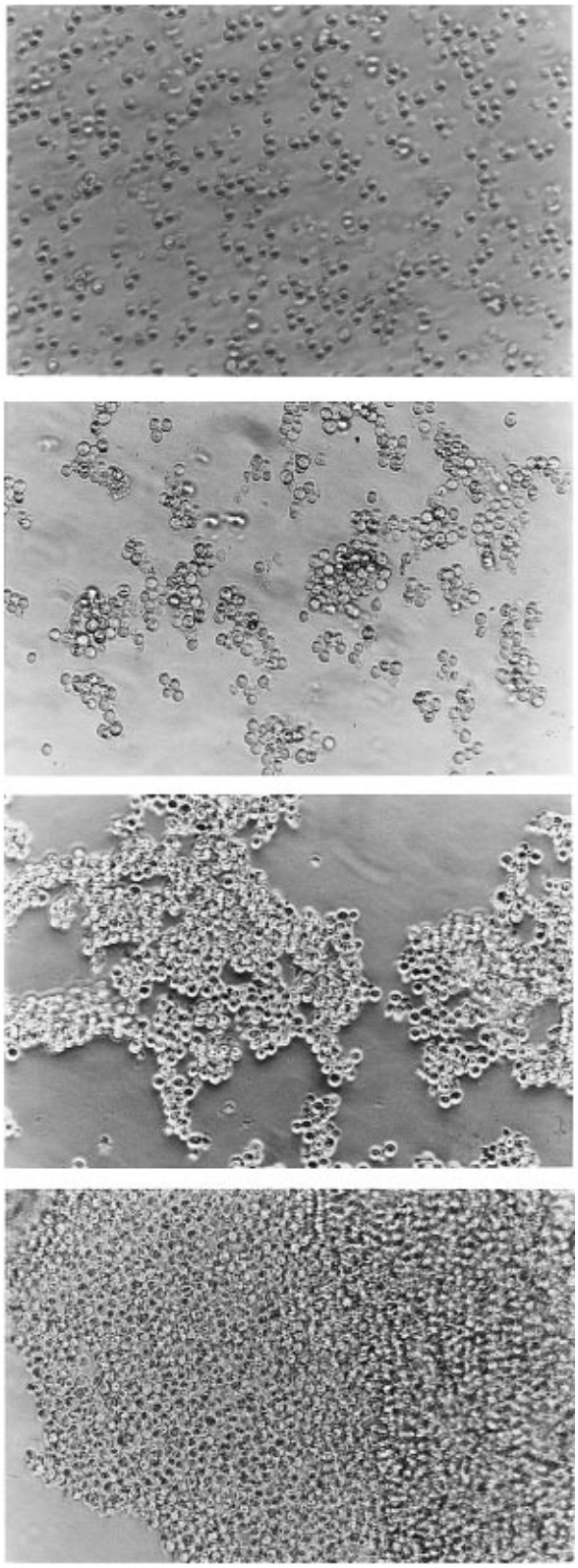

Figure 6. Cell cultures of SupT1 cells in a solution of (a) 0 g L^{-1}, (b) 1.25 g L^{-1}, (c) 2.5 g L^{-1}, and (d) 5 g L^{-1} α,ω-cholesterol-modified poly(oxyethylene); the number of cells in the samples is always approximately the same. The mean diameter of the cells is 10 μm (magnification 200×).

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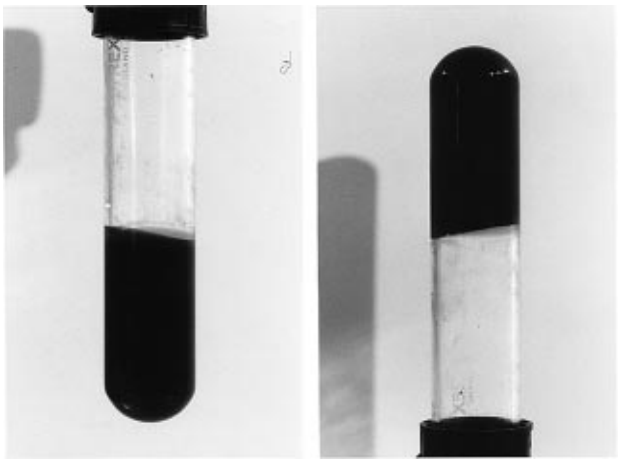

Figure 7. "Cross-linked" human blood (+citrate buffer) containing 5 g L^{-1} α,ω-cholesterol-modified poly(oxyethylene).

connection: formation of the first polymer bridge increases the probability for the formation of the next one.

To build up a network structure formed by the polymer together with the cells, one basic requirement is, however, that the cells should not be impaired too much by the insertion of the cholesterol end groups into the cell membrane. This could be shown for example with human lymphoblastoid cells (SubT 1), which could be metabolically labeled with fluorescent fatty acids in the presence of the polymer; i.e., the polymer does not destroy or kill the cells or suppress their metabolism.

Light microscopy shows that the polymer induces the formation of cell clusters (Figure 6). Experiments with pure unmodified poly(oxyethylene) do not lead to comparable effects at the low concentrations used, in agreement with the reported rather high polymer concentrations necessary to induce cell aggregation and fusion [see for example ref 4]. This indicates that indeed the cholesterol end groups of the polymer are necessary for this artificial cell colony formation and that—in agreement with the considerations above—the underlying mechanism is presumably the insertion of the polymer end groups into the cell membrane. With increasing polymer concentration the clusters of individual cells grow, and finally (Figure 6d) the formation of an "infinite" cluster throughout the whole sample is observed. This leads to a gelation of the whole nutritive solution whereby (a consequence of the size ratio) the elastically active skeleton is more or less exclusively formed by the cells. The cell-bridging polymer acts only as a sort of cell glue, inducing agglomeration to a tissue-like structure.

Figure 7 displays this gel formation with human blood. For concentrations larger than 5 g L^{-1} gelation of the whole sample within several minutes is observed even in presence of a citrate buffer to prevent natural blood coagulation. The resulting blood gels do not flow under the influence of gravity at least within a period of several days. Control experiments with unmodified poly(oxyethylene) did not lead to comparable modification of the rheological properties.

Conclusions

It has been demonstrated that α,ω-hydrophobically modified water-soluble polymers are able to interconnect vesicles and living cells probably by inducing their hydrophobic anchor groups to move into the lipid bilayer. The resulting vesicle−polymer networks represent new hydrogels with interesting structural and mechanical properties. The interactions between our synthetic polymer and living cells lead to at least qualitatively analogous results and probably open new possibilities in, for example, immobilization of cells at solid interfaces, blood coagulation, or culturing of artificial tissue. Nevertheless, this paper represents only a first description of these new materials which are the result of an interaction between synthetic polymers and biologically relevant structures. To understand both the underlying mechanisms and the physical properties, a multitude of further investigations are necessary.

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