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Phagocytosis by cultured mouse peritoneal macrophages of microspheres coated with perfluoroalkylated telomeric surfactants derived from tris(hydroxymethyl)aminomethane

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Abstract

A series of perfluoroalkylated telomeric surfactants derived from tris(hydroxymethyl)aminomethane (Tris), with adjustable hydrophilic character, has been studied with respect to their ability to influence the in vitro uptake by mouse peritoneal macrophages of polystyrene microspheres coated with these surfactants. The perfluoroalkylated surfactants were compared to hydrocarbon analogues. The nature and length of the hydrophobic tail, and the number of tris(hydroxymethyl) groups present in the molecule (3–20) are shown to affect the phagocytic uptake. Phagocytic uptake in the presence of serum is lower for the more hydrophobic C_8F_{17} tail than for the shorter C_6F_{13} tail. Phagocytosis is of lesser extent with the C_8F_{17} chain than with its hydrogenated analogue C_8H_{17}. An increase in the number of tris(hydroxymethyl) groups first causes a decrease in phagocytic uptake. A minimum is reached for approximately seven tris(hydroxymethyl) groups, after which uptake increases again. The combination of the characteristics favorable to a decrease in phagocytic uptake is present in F8TAC7 (C_8F_{17} hydrophobic tail and seven tris(hydroxymethyl) groups). In the presence of serum this surfactant gave the lowest phagocytic uptake of all the series of surfactants investigated. The uptake was then comparable to that observed for microspheres coated with egg yolk phospholipids.

Key words: Phagocytosis; Peritoneal macrophage; Polystyrene microsphere; Perfluoroalkylated telomeric surfactant; Zeta potential; Hydrophilicity; Surface mobility

1. Introduction

The achievement of an intravenously injectable oxygen-delivery system which could, among other applications, serve as a temporary substitute for blood, is topical. Considerable progress has recently been achieved in this respect with the formulation of stable, highly concentrated, and hence effective oxygen-carrying fluorocarbon emulsions (Riess, 1991, 1992). Further desirable improvements include the obtaining of emulsions with prolonged intravascular persistence. The search for more stable emulsions

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has led to the development of new, more effective surfactants and/or cosurfactants with increased fluorophilic character compared to the two surfactants, Pluronic F-68® (a polyoxypropylene polyoxyethylene block polymer) and egg yolk phospholipids (EYP), used in the currently developed emulsions (Krafft et al., 1991; Riess et al., 1992a,b, 1993a; Riess and Greiner, 1993). The desired increase in fluorophilic character was achieved by introducing a perfluoroalkylated tail into the surfactant. Such surfactants allowed the preparation of emulsions stable at room temperature with prolonged shelf-life (Riess and Postel, 1992; Riess et al., 1992a,b, 1993a,b; Riess and Greiner, 1993). More generally, such surfactants, due to their considerable effectiveness and high efficiency in terms of surface activity, and to their aptitude to create a hydrophobic and lipophobic barrier within a bilayer membrane (Riess et al., 1991, 1993a), are expected to play a role as components of vesicles and other assemblies destined for drug delivery (Cornelus et al., 1993).

The fate of fluorocarbon emulsions in the organism is that of all particulate-based carriers. After intravascular injection, fluorocarbon droplets are recognized as foreign particles by the reticuloendothelial system (RES) and progressively removed from the blood by liver and spleen macrophages (Geyer, 1983; Bradfield, 1984). Recognition of the particles is largely dependent on the nature of the surfactant film which surrounds each droplet, and hence on its components. Previous work indicates that the uptake of particles is dependent upon their size (Kawaguchi et al., 1986; Holmberg et al., 1990) and serum opsonic factors (Davis and Illum, 1983, 1988; Moghimi and Patel, 1989), and can be reduced by avoiding net surface charge (Stossel and Mason, 1972; Roerdink et al., 1983; Dijkstra et al., 1985), increasing surface hydrophilicity (Davis and Hansrani, 1985; Davis et al., 1985; Klibanov et al., 1990; Senior et al., 1991) and increasing membrane rigidity (Allen et al., 1989, 1991). The surfactants investigated in this paper had a priori all the attributes desirable for preventing rapid adsorption of plasma proteins on microspheres and subsequent phagocytosis: absence of net charge, highly hydrophilic character, and rigidity through

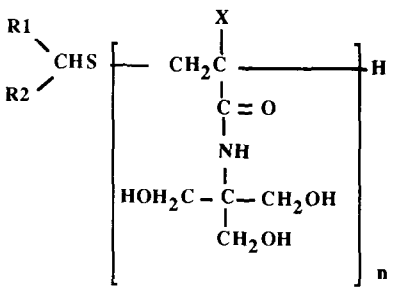

Fig. 1. Structure of the surfactants derived from tris(hydroxymethyl)aminomethane. R1 and R2 represent the hydrophobic chains; *n*, number of tris(hydroxymethyl)aminomethane groups in the molecule; X, H or CH_3.

probable hydrogen bondings. More recent data on liposomes indicate, however, that these principles need to be revised. Indeed, it has been shown that intravascular particle longevity is not necessarily destroyed by net charges or by lipid membrane fluidity and is not a direct consequence of high surface hydrophilicity either. Other factors, including steric forces and surface mobility, must be taken into consideration (Cevc et al., 1990; Blume and Cevc, 1992, 1993).

In this paper, we examine the modification of in vitro phagocytic uptake of microspheres coated with a new family of perfluoroalkylated telomeric surfactants (acrylates and metacrylates) derived from tris(hydroxymethyl)aminomethane (Tris) (Pavia et al., 1991, 1992). The characteristics of these surfactants can be adjusted over a wide range by changing the nature of the hydrophobic chain and the number of Tris groups present in the molecule (Fig. 1 and Table 1). Pluronic F-68 and EYP, which are present in the first genera-

Table 1
Surfactants investigated in this study (see Fig. 1)

Surfactants	R1	R2	X	*n*
F6TAC6.5	C_6F_{13}	H	H	6.5
F6TAC12	C_6F_{13}	H	H	12
F8TAC3.5.	C_8F_{17}	H	H	3.5
F8TAC7	C_8F_{17}	H	H	7
F8TAC12	C_8F_{17}	H	H	12
H8TAC7	C_8H_{17}	H	H	7
MH8TAC5.5	C_8H_{17}	H	CH_3	5.5
H11H12TA20	$C_{11}H_{23}$	$C_{12}H_{25}$	H	20

tion of fluorocarbon emulsions, as exemplified by Fluosol (Naito and Yokoyama, 1978, 1981), were used as reference compounds to assess the effects of the new Tris-derived surfactants on phagocytosis.

In this study we used mouse peritoneal macrophages and polystyrene microspheres coated with surfactant. The use of calibrated microspheres eliminated the particle size factor. Proper coating of the microspheres by the surfactant was demonstrated by microelectrophoresis (Müller, 1991). Data obtained through such an in vitro approach have been shown to correlate well with those obtained from in vivo phagocytic uptake experiments by the reticuloendothelial system (Illum et al., 1986).

2. Materials and methods

2.1. Materials

The perfluoroalkylated surfactants and their hydrocarbon analogues (Table 1) were synthesized by the Laboratoire de Chimie Bioorganique in Avignon. Fluorescent polystyrene microspheres (2.5% suspension, 1.07 μm mean diameter, labelled with fluorescein isothiocyanate) were obtained from Polyscience Inc. Photon correlation spectroscopy was employed to verify the particle sizes (Coulter model N4MD, Coulter Electronics, Hialeah, U.S.A.). Egg yolk phospholipids came from Asahi Glass; Pluronic F-68 was obtained from Serva. The products for cell culture were purchased from Gibco.

2.2. Coating of the particles

A suspension of microspheres (2.5% w/v) was mixed with an equal volume of surfactant solution (1–2% w/v) and incubated for 24 h at room temperature. For each surfactant the concentration was chosen to be on the plateau of the adsorption isotherm. The surface charge on the uncoated and coated microspheres was determined using laser Doppler anemometry (Zetasizer 3, Malvern Instruments, Malvern, U.K.). The solution of microspheres was diluted in 10–2 N KCl solution at pH 7 and 25°C. The electrophoretic mobility and the zeta potential obtained were the mean of three measurements.

2.3. Phagocytosis experiments

Male mice, weighing 20–22 g (IOPS, OF1), were killed by dislocation of the neck, the peritoneal wall of each animals was exposed and 5 ml of tissue culture medium E199 containing 10% swine serum, sodium bicarbonate, antibiotic (penicillin, streptomycin) and heparin (Illum et al., 1986) were injected into the peritoneal cavity as well as a smaller volume of sterile air. The peritoneal wall was gently massaged. The resulting macrophage-containing suspension was withdrawn and collected in a sterile container at 0°C. The exudates from several animals were collected and pooled. The cells were counted using a Malassez cell counter and the viability (of the order of 95%) of the macrophages was assessed by the Trypan Blue exclusion test. Exudates containing too many red blood cells were not used. The macrophage suspension was adjusted to 1×10^6 cells in 30 mm Petri dishes. After 3 h the plates were then washed once with sterile PBS to eliminate non-adherent cells, and 1.25 ml of the same medium with 10% swine serum, sodium bicarbonate and antibiotic was added. The plates were incubated in 95% air/5% CO_2 at 37°C for 24 h, after which the medium was removed and the cells washed once with sterile PBS. 2.5 ml of the same tissue culture medium, with and without 10% swine serum, containing the uncoated or coated microspheres (the number of particles was adjusted to 20 particles/macrophage) were added to each plate and the plates were incubated at 37°C in 95% air/5% CO_2 for 1 h. The medium was then removed, the plates washed twice with sterile PBS, and the cells stained with Wright's colorant.

The number of microspheres phagocytosed per macrophage was estimated using fluorescence microscopy at a magnification of 500 ×. The experiments were performed three times in triplicate. The results were expressed as a relative percentage of phagocytosis, i.e., the percentage of uptake with respect to that of the uncoated mi-

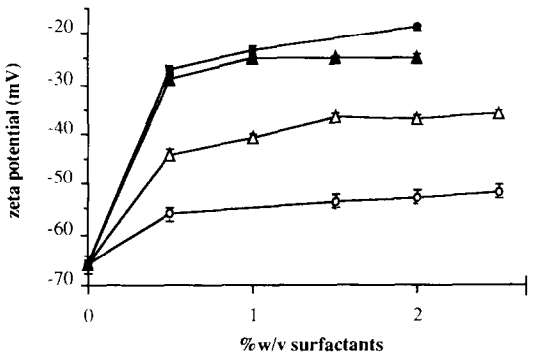

Fig. 2. Evolution of zeta potential with the percentage (w/v) of surfactant: (●) H11H12TAC20, (▲) F8C11TAC20, (△) H10TAC9, (○) H10TAC5.

crospheres (taken as 100%). The mean of the relative percentage of phagocytosis is given for each surfactant as well as the standard error of the mean.

3. Results

3.1. Surface charge

The particle surface charge as measured by laser Doppler anemometry as a function of increasing concentrations of surfactant showed that above 1% w/v a plateau is reached for most of the coating materials tested (Fig. 2). Therefore, the concentration chosen (1–2% w/v) was deemed to be largely sufficient to ensure complete coating of the microspheres. Microelectrophoresis experiments, with or without serum, demonstrated a decrease in electrophoretic mobility for all the coating materials tested (Table 2) (Davis et al., 1986).

In the absence of serum, when the length of the perfluoroalkyl chain (C_6F_{13} or C_8F_{17}) was held constant and the number of Tris groups increased, the electrophoretic mobility (from which the zeta potential is calculated) decreased (Fig. 3). Not unexpectedly, the lowest electrophoretic mobility was obtained with the surfactant having the largest number of Tris groups, i.e., H11H12TAC20 (see Table 1 for notation of compounds). The microspheres coated with this surfactant or with Pluronic F-68 showed the same zeta potential.

Table 2
Electrophoretic mobilities (EPM) and zeta potential (ZP) for microspheres uncoated and coated with different materials in 10^{-2} N KCl, pH 7, 25°C

Coating materials	EPM (μm cm S^{-1} V^{-1})		ZP (mV)	
	Without serum	With swine serum	Without serum	With swine serum
None	−4.612	−1.043	−65.9	−14.9
EYP	−3.398	−0.91	−48.6	−13
Pluronic F-68	−1.134	−0.644	−16.2	−9.2
F6TAC6.5	−3.447	−0.953	−49.3	−13.6
F6TAC12	−2.676	−0.977	−38.3	−14
F8TAC3.5	−3.508	−1.012	−50.16	−14.5
F8TAC7	−2.912	−0.957	−41.6	−13.7
F8TAC12	−2.265	−0.966	−32.4	−13.8
H8TAC7	−3.369	−0.89	−48.2	−12.7
MH8TAC5.5	−2.168	−0.926	−31	−13.2
H11H12TAC20	−1.323	−1.004	−18.9	−14.3

In the presence of serum, the zeta potential measured was similar for uncoated microspheres and microspheres coated with EYP or with Tris-derived surfactants. On the other hand, a large decrease in zeta potential was observed in the presence of serum with Pluronic F-68.

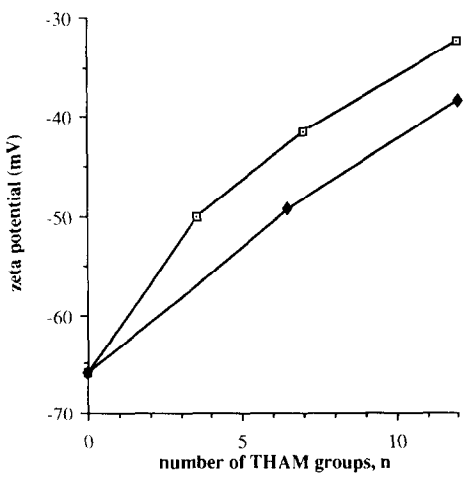

Fig. 3. Variation of zeta potential as a function of n (in 10^{-2} N KCl, pH 7, 25°C) for a given perfluoroalkylated tail: (□) C_8F_{17}; (♦) C_6F_{13}.

3.2. Effect of the nature and length of the hydrophobic tail

If one compares the phagocytosis of microspheres with and without serum for a series of surfactants (Fig. 4), one can see that the presence of serum had no significant effect on the relative percentage of phagocytic uptake of microspheres coated with EYP. On the other hand, the presence of serum resulted in a large increase in this percentage for microspheres coated with Pluronic F-68. For the microspheres coated with Tris-derived surfactants the addition of serum resulted in differences depending on the length and nature of the hydrophobic tail. When the surfactant had a hydrogenated hydrophobic tail (H8TAC7, MH8TAC5.5) the presence of serum enhanced the phagocytic uptake. The same behaviour was observed with the compound containing two hydrogenated hydrophobic tails (H11H12TAC20). On the other hand, the fluorinated surfactants displayed two distinct behaviours depending on the length of their hydrophobic tail. For the C_6F_{13} tail the same results as for hydrogenated hydrophobic tails were observed while with the longer perfluoroalkylated tail C_8F_{17} the presence of serum resulted in a reduction in the relative percentage of phagocytosis.

When the microspheres with H8TAC7 and F8TAC7 (same number of Tris groups and same hydrophobic tail length, but hydrogenated vs fluorinated) were compared, there was no difference in phagocytic uptake without serum, however, the F8TAC7-coated microspheres underwent lower phagocytosis than those coated with H8TAC7 in the presence of serum.

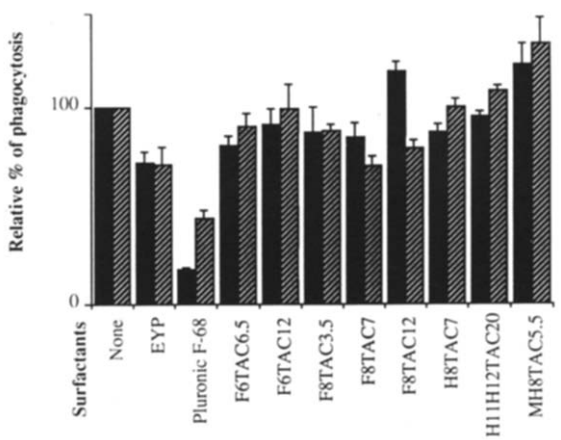

Fig. 4. Relative percentage of phagocytosis of surfactant-coated microspheres: (filled bars) without serum, (hatched bars) with serum (number of experiments = 3).

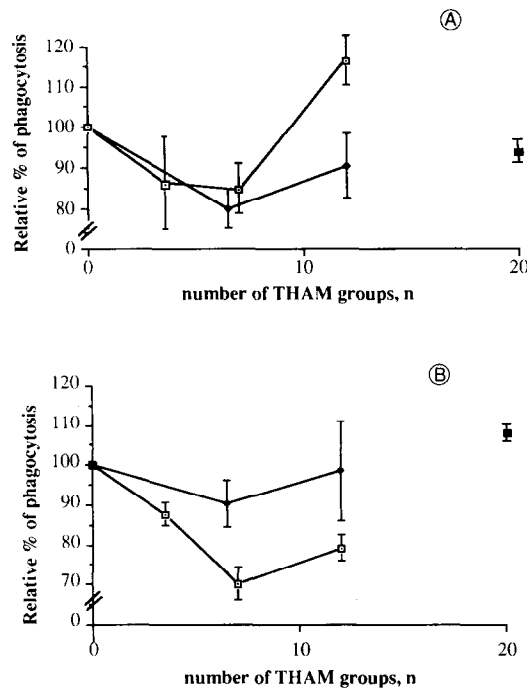

Fig. 5. (A) Effect of the value of n in the phagocytic uptake of surfactant-coated microspheres without serum; (♦) F6TAC, (□) F8TAC, (■) C11C12TAC20. (B) Effect of the value of n in the phagocytic uptake of surfactant-coated microspheres with serum; (♦) F6TAC, (□) F8TAC, (■) C11C12TAC20.

3.3. Effect of the surfactant's hydrophilic character (number of tris(hydroxymethyl)aminomethane groups)

To study the effect of the number of Tris groups, we used a surfactant with a large n value, namely, H11H12TAC20 which has two hydrophobic hydrocarbon tails and 20 Tris groups. Surprisingly, the H11H12TAC20-coated microspheres generated phagocytic uptake close to that found for uncoated microspheres.

The effect of the number of Tris groups (n) was also examined for the same perfluoroalkylated tail, C_6F_{13} or C_8F_{17} (Fig. 5A and B). In

both cases the experiments, with and without serum, indicated that when the number of Tris groups increases the phagocytic uptake first decreases, then reaches a minimum when n is around 7 after which the uptake, unexpectedly, increases again. The presence of a minimum is more pronounced in the case of the longer perfluoroalkyl chain.

The experiments with microspheres coated with MH8TAC5.5 show that the presence of a methyl group on the tris(hydroxymethyl)aminomethane group dramatically increases the phagocytic uptake, irrespective of whether or not serum is present.

4. Discussion

One of the major challenges in developing injectable emulsions, vesicles and other particulate-type carriers for biomedical applications is to achieve proper control and modulation of their phagocytosis. A range of parameters (size, charge, rigidity, hydrophilicity, surface microstructure, etc.) influence the recognition of particles by the cells and their phagocytic uptake by the RES. Many of these parameters are determined by the surfactants utilized. Surfactants largely determine both the physical properties of the dispersion of particulates and the nature and structure of the surface particles. Unfortunately, the question of which parameters and characteristics are most favorable to prolonged intravascular persistence and targeted biodistribution is still a matter of debate (Cevc et al., 1990; Blume and Cevc, 1992).

In our experiments, the surface charge differences measured in the presence of serum on uncoated microspheres and on microspheres coated with EYP or Tris-derived surfactants were probably not large enough to result in significant effects with respect to particle recognition by the macrophages. On the other hand, in the absence of serum, when one compares the results with H11H12TAC20 and Pluronic F-68, one observes that microspheres having similar zeta potentials can generate very different phagocytosis and that a decrease in surface charge does not systematically result in a decrease in phagocytic uptake. These observations are in agreement with data indicating that surface charge is not necessarily a determining factor in particle phagocytosis (Van Oss, 1978).

Our experiments with serum indicate lower phagocytosis (43.3%) with a Pluronic F-68 coating than what was observed by Illum et al. (1986) with 5 μm polystyrene microspheres (94.5%). Our results are, however, in accordance with those obtained by Müller et al. (1992) with smaller polystyrene microspheres. The size of the microspheres is likely to be responsible for these differences.

It has been well established that microspheres coated with hydrophilic materials of the polyoxyethylene type are taken up less rapidly by peritoneal macrophages in vitro, and by the reticuloendothelial system in rabbits in vivo (Davis and Hansrani, 1985; Klibanov et al., 1990; Carstensen et al., 1991). This has led to the opinion that hydrophilic surface coating in general would delay phagocytosis. We thought, therefore, that a larger number of Tris groups (n), by providing a large number of hydroxylic groups at the particle's surface, could strongly reduce phagocytic uptake. Our experimental data do not confirm this hypothesis. Under the conditions utilised, whether with or without serum present, only a minimum in phagocytosis was observed for n around 7, and the percentage of phagocytosis never fell below 70% with respect to uncoated microspheres. A larger number of polar groups, although resulting in a decrease in surface charge and in an increase in hydrophilic character, does not limit phagocytic uptake.

One possible reason for this disappointing result could be that the numerous hydrogen bonds which are likely to occur between the large number of carboxyl, amine and hydroxyl functions present in the hydrophilic head: 'neutralize' these functions in terms of availability for hydrogen bonding with external water molecules, thus decreasing the hydrophilic character of the surface (Woodle and Lasic, 1992), and render the surface's structure more rigid. Surface mobility was indeed also identified to be a requisite for reduced opsonization and recognition (Blume and Cevc, 1993).

Phagocytosis is dramatically increased when a methyl group is present on the Tris group. This methyl is expected to increase the lipophilic character of the coating layer and hence facilitate the adsorption of serum components on the microspheres' surface and subsequent recognition by macrophages.The fluorinated chains appear to have a favorable influence on phagocytic uptake in the presence of serum. In our experiments with serum an increase in fluorophilic character, with the surfactant having a C_8F_{17} tail, results in a decrease in the relative percentage of phagocytosis. This behaviour may be related to the increase in lipophobic character developed inside the surfactant film, which may hinder the penetration of serum proteins into this film.

For the perfluoroalkylated surfactants the largest decrease in phagocytic uptake in the presence of serum was achieved by coating the particles with F8TAC7 (70%). This relative percentage of phagocytosis with serum was then very similar to that obtained with EYP but significantly greater than with Pluronic F-68. In the series of surfactants investigated, F8TAC7 has an optimal value of n, i.e., balance in terms of hydrophilic character and surface mobility, and a long perfluoroalkylated chain which induces a lipophobic character inside the surfactant coating. Previous studies concerning the biocompatibility of this surfactant and hemolytic activity (Zarif et al., 1993) indicate that F8TAC7 could be interesting as a component of fluorocarbon emulsion, vesicles and other drug delivery systems.

5. Acknowledgements

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