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Stabilization of Indocyanine Green by Encapsulation within Micellar Systems

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Abstract: Indocyanine green (ICG) is a fluorescence dye that is widely used for near-infrared imaging. Application of this dye is limited by its numerous disadvantageous properties in aqueous solution, including its concentration-dependent aggregation, poor aqueous stability *in vitro* and low quantum yield. Additionally, ICG is highly bound to nonspecific plasma proteins, leading to rapid elimination from the body with a half-life of 3−4 min. In this study, encapsulation of ICG within various micellar systems was investigated with the aim of overcoming these limitations. The aggregation behavior of different aqueous ICG formulations was studied using cryogenic transmission electron microscopy (cryo-TEM) and absorption spectroscopy. The micellar systems were characterized by their optical properties, particle size distribution, zeta potential and hemolytic activity. Encapsulation efficiency was determined using analytical ultracentrifugation. The best results were achieved for ICG encapsulated within aqueous Solutol HS 15 micelles. This formulation exhibited a lower aggregation behavior, a 3-fold increased quantum yield and high aqueous stability (over 4 weeks) compared to free aqueous ICG. The micelles were found to have an average diameter of 12 nm and a zeta potential close to zero (−2.1 ± 1.7 mV). Encapsulation efficiency of ICG was high at 95%. The formulation did not display significant hemolytic activity. Consequently, Solutol HS 15 micelles are suitable nanocarriers for ICG which improve the optical properties and stability of the dye.

Keywords: Indocyanine green; aggregation; micelles; stability; hemolysis

Introduction

Indocyanine green (ICG), a water-soluble, amphiphilic tricarbocyanine dye, offers a broad range of applications due to its low toxicity[1] and capacity to absorb and emit in the near-infrared spectral range.[2] Since human tissue has the lowest absorption coefficient in the near-infrared part of the spectrum, ICG can be used for many therapeutic and diagnostic applications such as cardiac output, liver function

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and fluorescence angiography.[3−6] However, several physicochemical characteristics limit the application of ICG *in vitro* and *in vivo*.

The optical properties of ICG strongly depend on the dye concentration and the solvent. In aqueous solution, ICG starts to form dimers and oligomers at dye concentrations over 3.9 mg/L, leading to nonapplicability of Beer−Lambert's law.[7,8] At concentrations greater than 10^3 mg/L ICG, the dye molecules form large J-aggregates, which induce a new absorption maximum at $\lambda = 900$ nm after seven days.[2,9−11] The presence of aggregates causes self-quenching of the dye and hence reduces fluorescence. Additionally, ICG undergoes degradation in aqueous media resulting in a simultaneous loss of absorption and fluorescence. This is due to saturation of the double bonds in the conjugated chain, leading to the formation of leucoforms. The presence of solvent radicals and ions, which in the case of aqueous solutions are provided by water, activates this degradation process.[12,13] The half-life of 1 mg/L ICG in aqueous solution when stored in the dark at 22 °C is only 16.8 ± 1.5 h.[13] Light exposure and physiological temperature accelerate the decay process.[2,12,14,15] Due to its amphiphilic character, ICG can interact with both lipophilic and hydrophilic molecules. In plasma, ICG binds almost completely (98%) to most of the major plasma proteins such as albumin, globulins and lipoproteins.[11,16−18] After binding to plasma proteins, ICG is excreted exclusively by means of the liver. The precise uptake of ICG from the plasma by the hepatic parenchymal cells is ambiguous. It is assumed that a dissociation of the protein ICG complex occurs and that the ICG molecule is carried across the sinusoidal membrane via active carrier mediated transport. ICG is then vesicularly transported through the hepatocyte, and is secreted entirely into the bile by multidrug related protein 2 and exocytosis.[19,20] Only negligible uptake of the dye occurs in peripheral tissue, and excretion by the kidney does not occur.[21] This behavior of ICG causes a rapid elimination from the bloodstream, with an initial half-life of 3−4 min, followed by a slower half-life of approximately 1 h at lower concentrations.[13,22]

Various nanosized carriers for ICG have been investigated to overcome the high degradation rate and the short plasma half-life.[23−25] Poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles and nanoparticle-assembled capsules (NACs) containing ICG, with approximate diameters of 0.4 to 1.0 μm, showed increased stability and plasma half-life compared to the free ICG solution.[23,24,26,27] All of these nanosized ICG formulations displayed an equivalent or even lower fluorescence intensity compared to free ICG. Oil in water emulsions containing ICG were also found to show an increased blood

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half-life and absorption capacity, but the *in vitro* stability was not examined.[28] Recently, Rodriguez et al. prepared poly(styrene-*alt*-maleic anhydride) block-poly(styrene) micelles with an approximate diameter of 55 nm to entrap hydrophobic ICG-tetrabutylammonium iodide within the micelles using a solvent evaporation method.[25] Application of a micellar formulation has several advantages over PLGA nanoparticles or NACs. The size of surfactant micelles, approximately 10−100 nm, is within the range that allows extravasation and permeation into tissues and yet is small enough to avoid clearance by the reticuloendothelial system (RES).[29−31] The prolonged circulation in the bloodstream is necessary to achieve a passive accumulation in tumors or sites of inflammation via the enhanced permeation and retention (EPR) effect.[32−34] Incorporating ICG into micelles using a simple aqueous-based preparation method would be a beneficial way to prepare an application system that protects the dye from degradation and binding to plasma proteins.

In this study, a variety of novel aqueous micellar formulations were investigated as vehicles for ICG. In addition to several well-known surfactants, such as Tween 80, Pluronic F 68, Cremophor RH 40 and Solutol HS 15, two sugar-based surfactants (Ryoto sugar ester L 1695 and S 1170) and mixed micelles containing glycocholic acid and phosphatidylcholine were investigated. Most of the surfactants used were materials approved for parenteral administration. The two sugar-based surfactants were chosen as an alternative to these surfactants. They are not approved for parenteral administration so far, but have been used for many years in the cosmetic and nutrition industry. They are promising surfactants for parenteral use, because of their low toxicity, biodegradability and excellent emulsifying properties. Additionally, they are nonionic and display a lower temperature sensitivity in comparison to the PEG surfactants.[35] The suitability of these sugar-based surfactants as parenteral excipients was investigated. The micelle formation and encapsulation of ICG occurred via the standard direct dissolution method, in which the surfactants and ICG were simply added to the aqueous medium while stirring. Concentration-dependent dimerization of ICG, both in aqueous solution and in the micellar formulation, was investigated. The time-dependent formation of J-aggregates at high ICG concentration (10^3 mg/L) was also examined. The formulations were characterized with regard to their optical properties, micellar size distribution, zeta potential and encapsulation efficiency. Both aqueous and thermal stability were examined, and the *in vitro* hemolytic activity was determined.

Experimental Details

Materials. Indocyanine green (Cardiogreen) free of sodium iodide was purchased from Fluka (Taufkirchen, Germany). Polyoxyethylene 20 sorbitan monooleate (Tween 80), polyoxyethylene-polyoxypropylene block copolymer (Pluronic F 68) and glycocholic acid were obtained from Sigma Aldrich (Taufkirchen, Germany). Polyoxyethylene esters of 12-hydroxystearic acid (Solutol HS 15) and polyoxyl 40 hydrogenated castor oil (Cremophor RH 40) were donated by BASF (Ludwigshafen, Germany). Lipoid S 100, a phosphatidylcholine from fat-free soybean lecithin, was obtained from Lipoid GmbH (Ludwigshafen, Germany). Sucrose esters of laurate (Ryoto sugar ester L 1695) and sucrose esters of stearate and palmitate (Ryoto sugar ester S 1170) were kindly provided by Mitsubishi-Kagaku Foods Corporation (Tokyo, Japan).

All other chemicals were of analytical grade and used as received, unless stated otherwise. Water used throughout was distilled and deionized in a Milli-Q water purification system (Millipore, Bedford, U.K.).

Preparation of ICG-Incorporated Micelles. Micelles were prepared by the direct dissolution method, in which the surfactants were simply added to the distilled water at room temperature at various concentrations, ranging from 0.05 to 100 mg/mL. In the case of the mixed micelles, 10 mL water was heated to 60 °C and 540 mg of glycocholic acid and 900 mg of phosphatidylcholine or 100 mg of sucrose ester S 1170 were suspended and homogenized for 1.5 min using a SilentCrusher S (Heidolph Instruments, Germany) set at 75,000 rpm. The pH value was adjusted to 6.5 using sodium hydroxide, and the resulting solutions were allowed to cool to room temperature under continuous stirring. To achieve ICG-encapsulation, aliquots of a stock solution of ICG in distilled water (10^4 mg/L) were added to 5 mL of

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the micelle solutions to obtain final dye concentrations ranging from 0.1 to 15 mg/L. The resulting solutions were stirred at room temperature for an additional 30 min and were then stored in the refrigerator and used without further washing steps within 12 h.

Spectroscopic Measurements. Absorption and fluorescence measurements were performed on an Uvikon 933 double beam UV/vis spectrophotometer (Kontron Instruments, Germany) and a Spex Fluorolog-2 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) in acrylic cuvettes of 1 cm path length with plain micelles as reference. The ICG concentration used was 5 mg/L, at which no quenching effect of the dye occurred. The required surfactant concentration was determined by absorption spectroscopy, at which no more shifts in the absorption maximum peak occurred. This concentration was determined for all surfactants with 50 mg/mL. Each ICG formulation was excited at the wavelength found to show an absorption maximum in the absorption spectrum. Spectra were recorded from 500 to 900 nm. For comparison of fluorescence intensities of the different formulations, the spectra were corrected for the spectral response of the apparatus components.[36]

The fluorescence quantum yield (Φ) of ICG in different micelle formulations was determined relative to the quantum yield of ICG in dimethylsulfoxide (DMSO), $\Phi = 13\%$.[37] The quantum yield is defined as the ratio of the number of emitted photons to the number of absorbed photons. The area under the fluorescence curve and the absorbance at the excitation wavelength was determined and the quantum yield was evaluated using following equation:

$$\Phi_{\text{sample}} = \frac{\int f(\lambda)_{\text{sample}}}{(1 - 10^{-A})f} \tag{1}$$

where Φ_{sample} represents the quantum yield of the formulation; $f(\lambda)_{\text{sample}}$, the function of the emission curve; A, the absorbance of the sample at the excitation wavelength; and f, the correction factor.

The correction factor, f, was determined preliminarily from the reference ICG in DMSO as follows:

$$f = \frac{\int f(\lambda)_{\text{ICG/DMSO}}}{(1 - 10^{-A})\Phi_{\text{ICG/DMSO}}} \tag{2}$$

where $\Phi_{\text{ICG/DMSO}}$ represents the quantum yield of ICG in DMSO (13%); $f(\lambda)_{\text{ICG/DMSO}}$, the function of the emission curve; and A, the absorbance of ICG at the excitation wavelength.

Characterization of ICG Aggregates and ICG Micelles. Particle Size and Zeta Potential (ζ) Analysis. Particle size distribution and zeta potential were determined using a Zetasizer Nano ZS (Malvern Instruments, U.K.) equipped with a 4 mW HeNe laser beam with a wavelength of 633 nm and a scattering angle of 173°. The measurements were performed at 25 °C without further dilution of the samples. The CONTIN mode was used to extract intensity-weighted size distributions from the autocorrelation functions. This general-purpose program inverts the noisy autocorrelation functions by means of inverse Laplace transform whereby it produces several permissible solutions and chooses the most appropriate solution based on statistical evaluations.[38] The zeta potentials were automatically calculated using the Smoluchowski equation. The values are reported as the mean ± standard deviation based on three individual measurements performed in triplicate.

Cryogenic Transmission Electron Microscopy (Cryo-TEM). Cryo-TEM was performed on a Philips CM12 transmission electron microscope at 160 kV. Sample preparation was carried out at room temperature by placing 10 μL of the solution on a hydrophilized perforated carbon filmed grid. Excess fluid was blotted off and the grid was immediately vitrified in liquid ethane (−184 °C) using a standard plunging device. The specimens were imaged at a −175 °C sample temperature using low dose conditions at a magnification of 62000× and 100000× to minimize electron-beam radiation damage. A more detailed description can be found elsewhere.[39]

ICG Encapsulation Efficiency. The encapsulation efficiency of ICG into selected micelles was determined by analytical ultracentrifugation.[40] This method was used because ICG is a zwitterionic amphiphilic molecule, which strongly adsorbs to both hydrophilic and lipophilic surfaces. Using the conventional separation methods such as filtration or size exclusion chromatography were unsuccessful due to the strong interactions of ICG with the column or filter material. Sedimentation velocity was carried out at 25 °C using a Beckman Optima XL-A analytical ultracentrifuge equipped with absorbance and Rayleigh interference optics. The sedimentation behavior of ICG was investigated with the absorbance optic at 720 nm, where the dye shows a strong absorbance. The pure surfactants exhibited no optical absorbance at all and did not affect the measurement of ICG. The sedimentation behavior of the micelles were investigated with the Rayleigh interference optic at 675 nm. This technique relies on the fact that the velocity of light is decreased when passing through a region of higher refractive index. The advantages of this method are that it is applicable to materials with little optical absorbance and, additionally,

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is not compromised by components at low concentrations within the solvent, which have relatively high absorbance.[41] The concentrations used were 10 mg/L ICG and 10 mg/mL surfactant. The experiments were performed at 45,000 rpm in a double sector cell of 20 mm path length. Data were collected at 720 nm (absorbance) and 675 nm (interference). The raw data were analyzed within the range 6.3−6.9 cm cell radius for the first 131 scans.

Aqueous Stability of ICG Micelles. The influence of aqueous media on degradation of ICG was determined for ICG micelles and free aqueous ICG at 4 and 25 °C in the dark. The investigation on the stability at 25 °C in the dark was in accordance to the storage of the currently used ICG formulation. Additionally, the influence of temperature was investigated by determining the stability at 4 °C. The concentrations used for this experiment were 5 mg/L ICG and 50 mg/mL surfactant according to the spectroscopic measurements. Each sample was placed in a glass vial wrapped in aluminum foil to prevent light exposure. The absorbance at the monomeric absorption peak of each sample was measured at intervals, every other day during the first week and once a week for the next three weeks. Values shown represent the mean ± standard deviation of 6 individual batches.

Hemolytic Activity. Human heparinized whole blood was separated from plasma by centrifugation at 1000*g*, and the red blood cells (RBCs) were washed and diluted with isotonic PBS, pH 7.4. The micellar formulations were incubated with a 2% (v/v) RBC suspension at 37 °C for 1 h at different surfactant concentrations. The unlysed RBCs were removed by centrifugation, and the supernatant was analyzed by UV/vis spectroscopy at 540 nm. The percentage hemolysis was determined using the following equation:

$$\text{hemolysis } [\%] = \frac{A_{\text{sample}} - A_{0\%}}{A_{100\%} - A_{0\%}} \times 100 \qquad (3)$$

where A_{sample}, $A_{0\%}$ and $A_{100\%}$ represent absorbances of the sample, the negative control (PBS buffer) and the positive control (2% Triton X-100), respectively.

Results

Aggregation Behavior of ICG in Different Aqueous Formulations. The formation of J-aggregates of a 10^3 mg/L aqueous ICG solution stored at room temperature in the dark was observed by absorption spectroscopy and cryo-TEM. After storage for 2 weeks, a new absorption maximum at $\lambda = 900$ nm was observed (Figure 1). Spherical aggregates with an approximate diameter of 5 nm were observed in the corresponding cryo-TEM images (Figure 2A). Two weeks later, the spherical assemblies turned into large planar sheets having a width of about 3.6 nm (Figure 2B).

Concentration-dependent dimerization of ICG in distilled water was compared to ICG-encapsulated micellar formulations. For all solution types, low dye concentrations showed a linear correlation between concentration and absorbance in accordance with Beer−Lambert's law (Figure 3A). At higher concentrations, however, dimerization occurred, leading to nonlinearity. ICG encapsulated within surfactant micelles followed Beer−Lambert's law up to higher dye concentrations compared to free ICG (Figure 3B).

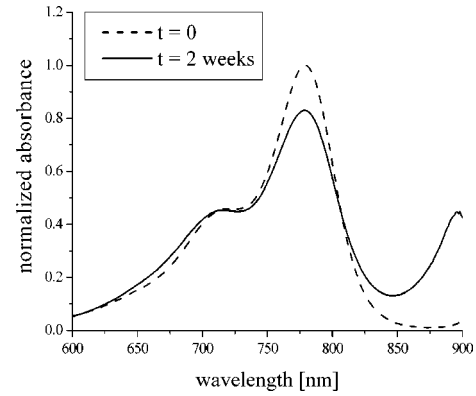

Figure 1. Absorption spectra of 10^3 mg/L aqueous ICG solution immediately after preparation (---) and after storage for 2 weeks at room temperature in the dark (—). After storage for 2 weeks, a new absorption maximum at $\lambda = 900$ nm was observed, indicating the formation of J-aggregates.

Characterization of ICG Formulations. Absorption and Fluorescence Spectroscopy. To analyze the absorption and fluorescence maxima and the quantum yield of ICG in different formulations, 5 mg/L ICG were dissolved in 50 mg/mL surfactant solution. The results are summarized in Table 1. Compared to ICG in water, the absorption and emission spectra of the micellar formulations were bathochrome-shifted by approximately 20−30 nm (Figure 4). Noticeably, ICG in Pluronic F 68 exhibited smaller shifts of only 5 nm (emission spectrum) and 9 nm (absorption spectrum).

The fluorescence quantum yields (Φ) of micellar encapsulated ICG were significantly enhanced, compared to the quantum yield in water (Φ = 2.58 ± 0.04%) and the reported quantum yield in plasma (Φ = 3.2%).[36] The highest effective increase was observed in Solutol HS 15 micelles (Φ = 8.33 ± 0.18%). The least effective quantum yield increase was observed for ICG in Pluronic F 68 (Φ = 3.16 ± 0.15%).

Size and Zeta Potential. Particle size, polydispersity index and zeta potential for various surfactant systems are outlined in Table 2. Dynamic light scattering was used to evaluate the intensity size distribution for plain micelles and selected micelles with ICG. The PDI values provide an indication of the particle size distribution (wide vs narrow). The PDI determined by the method of cumulants, adopts values between 0 and 1. Low values indicate narrow size distributions and higher values, a broad distribution. More precisely, a PDI < 0.1 depicts a monodisperse sample, 0.1 < PDI < 0.2 depicts a sample with a narrow particle size distribution

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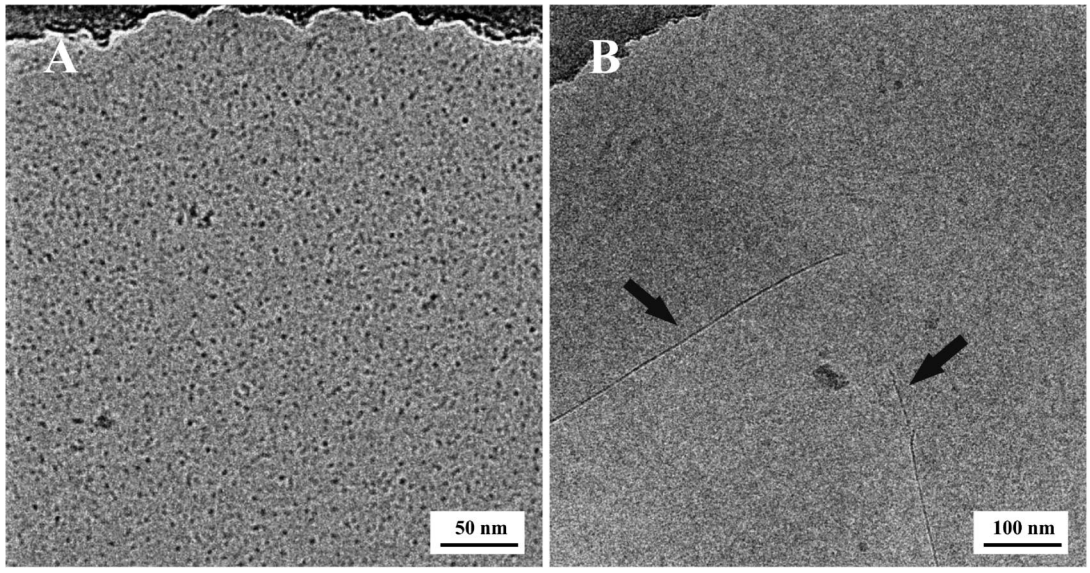

Figure 2. Cryo-TEM micrographs obtained from 10^3 mg/L aqueous ICG solution after storage at room temperature for 2 weeks (A) and 4 weeks (B). Specimens were imaged under low dose conditions at 100000× and 62000× magnification, respectively. After 2 weeks, spherical aggregates were present having an approximate diameter of 5 nm. After a further 2 weeks, these assemblies turned into large planar sheets having a width of about 3.6 nm (the edges are shown by black arrows).

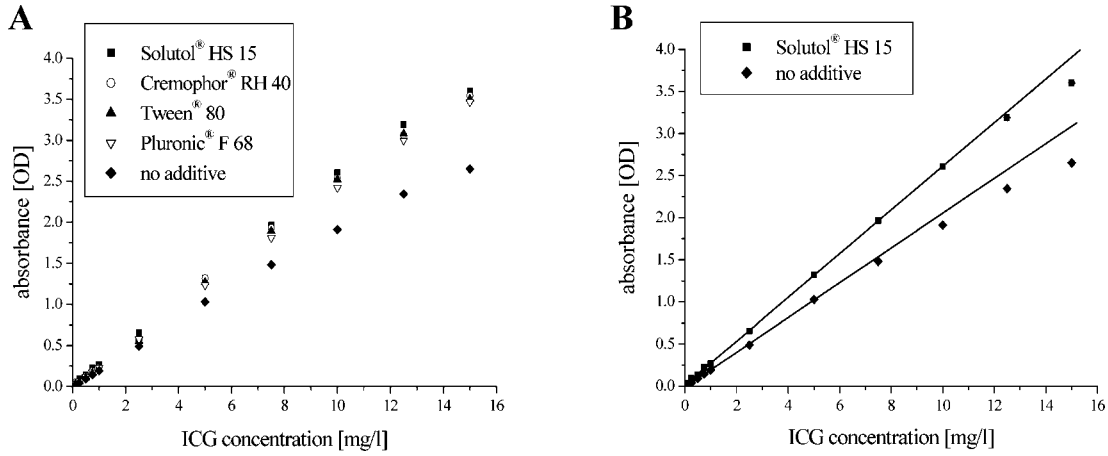

Figure 3. Concentration versus absorbance of ICG in different nonionic surfactant formulations (50 mg/mL) (A). Linear regression of free ICG and ICG in Solutol HS 15 (B). ICG within Solutol HS 15 micelles (■) reveals linearity up to a higher dye concentration compared to free ICG (◆). Data represent means of six experiments ± SD (depicted as bars). When no bar is visible, the SD was smaller than the symbol.

and 0.2 < PDI < 0.5 depicts a sample with a broad particle size distribution.[42]

Tween 80 and Cremophor RH 40 micelles showed small average hydrodynamic diameters (8.3 ± 0.1 nm and 13.7 ± 0.1 nm, respectively) with a narrow particle size distribution (PDI = 0.148 ± 0.007 and PDI = 0.110 ± 0.007, respectively). Solutol HS 15 micelles were monodisperse (PDI = 0.046 ± 0.005) with an average hydrodynamic diameter of 11.8 ± 0.1 nm, which is consistent with the data in the literature.[43] All other plain micelles were found to have broad or bimodal particle size distributions.

Zeta potentials of nonionic surfactant micelles were found to be around zero. Glycocholic acid had an average zeta potential of $\zeta = -37.3 \pm 8.0$ mV, whereas the zeta potentials of mixed micelles consisting of glycocholic acid and Lipoid S 100 or sucrose ester S 1170 were found to be $\zeta = -24.8 \pm 8.1$ mV and $\zeta = -24.7 \pm 5.0$ mV, respectively.

The addition of 10 mg/L ICG to selected plain micelles had no significant effect on particle size distribution and zeta potential (Table 3).

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Table 1. Surfactant Classification, Absorption and Fluorescence Maxima and the Quantum Yield of ICG (5 mg/L) in Different Micellar Formulations (50 mg/mL)

ICG formulation	surfactant classification	λ_{max} absorption[a] [nm]	λ_{max} emission[a] [nm]	Φ^b [%]
no additive		779	805	2.58 ± 0.04
Pluronic F 68	nonionic/HLB 29	788	810	3.16 ± 0.15
Tween 80	nonionic/HLB 15	798	825	6.77 ± 0.15
Cremophor RH 40	nonionic/HLB 14−16	798	822	7.22 ± 0.08
Solutol HS 15	nonionic/HLB 14−16	798	825	8.33 ± 0.18
sucrose ester L 1695	nonionic/HLB 16	802	830	7.95 ± 0.12
glycocholic acid (GA)	ionic	804	835	4.00 ± 0.11
glycocholic acid/sucrose ester S 1170	ionic	810	832	4.70 ± 0.13
glycocholic acid/Lipoid S 100	ionic	808	835	5.30 ± 0.16

[a] Absorbance and fluorescence measurements were performed in acrylic cuvettes of 1 cm path length with plain micelles as reference. [b] Fluorescence quantum yields (Φ) were determined relative to ICG in DMSO (Φ = 13%) and are listed as mean values ± SD of six experiments.

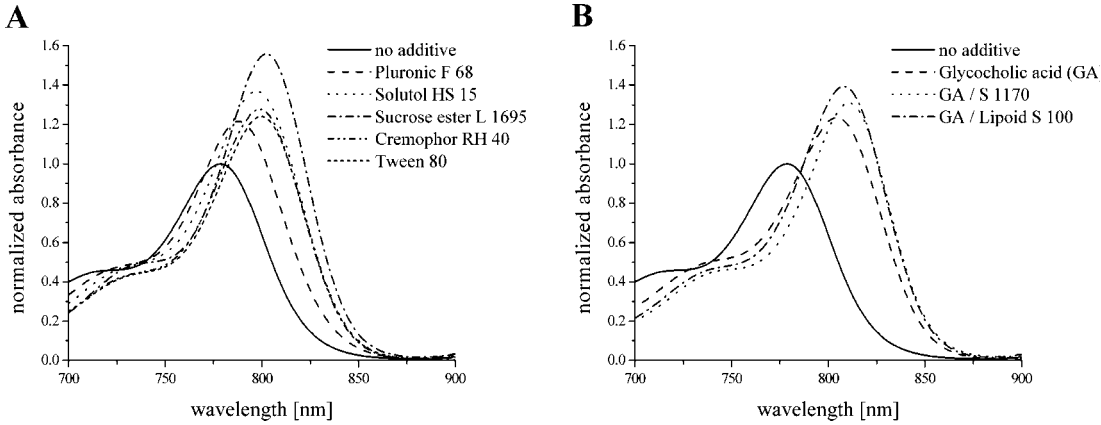

Figure 4. Absorption spectra of the investigated micellar systems (50 mg/mL) with ICG (5 mg/L). Nonionic ICG micelles (A) and ionic ICG micelles (B) displayed a bathochrome-shifted absorption spectrum and a greater absorbance in comparison to the free aqueous ICG solution.

Table 2. Hydrodynamic Diameter (d_H), Polydispersity Index (PDI), Zeta Potential and pH Value of Several Micellar Systems (100 mg/mL)

surfactant system	d_H[a,c] [nm]	PDI[a,c]	zeta potential[b,c] [mV]	pH value
Solutol HS 15	11.8 ± 0.1	0.046 ± 0.005	−2.1 ± 1.7	6.6
Cremophor RH 40	13.7 ± 0.1	0.110 ± 0.007	−1.6 ± 0.4	6.8
Tween 80	8.3 ± 0.1	0.148 ± 0.007	−3.8 ± 1.0	6.7
GA/sucrose ester S 1170	87.6 ± 0.1	0.204 ± 0.003	−24.7 ± 5.0	7.0
sucrose ester L 1695	7.0 ± 0.2	0.246 ± 0.003	−7.5 ± 1.6	6.8
Pluronic F 68	90.2 ± 82.0	0.323 ± 0.142	−3.3 ± 0.4	6.8
GA/Lipoid S 100	1.4 ± 0.04 (60%) 36.3 ± 4.0 (40%)	0.352 ± 0.022	−24.8 ± 8.1	6.7
glycocholic acid (GA)	1.1 ± 0.01 (10%) 125.0 ± 10.3 (90%)	0.558 ± 0.091	−37.3 ± 8.0	7.0

[a] Intensity-weighted mean hydrodynamic diameter and polydispersity index were determined by dynamic light scattering (DLS). Each measurement consisted of 30 runs, and the calculation mode used was CONTIN. [b] Zeta potential analysis was carried out using laser Doppler anemometry (LDA) with 30 runs per measurement. [c] The values represent the mean ± SD based on three individual measurements performed in triplicate.

ICG Encapsulation Efficiency. Sedimentation velocity experiments showed that ICG molecules only sedimented when they were encapsulated within the micelles. The mass of free dye molecules was too small to allow sedimentation. Absorbance and interference diagrams of ICG incorporated into micelles were found to have the same concentration profiles and nearly identical sedimentation coefficient distributions (Figure 5). Therefore, simultaneous sedimentation of dye and micelle indicated encapsulation. After sedimentation, the absorbance of residual ICG in the supernatant was determined and dye concentration was estimated by means of a calibration curve. The encapsulation efficiency was then determined by the ratio of incorporated ICG to initial ICG concentration. Five different surfactant systems with ICG

Table 3. Hydrodynamic Diameter (d_H), Polydispersity Index (PDI), Zeta Potential and pH Value of 100 mg/mL Surfactant Systems with 10 mg/L ICG

surfactant system + 10 mg/L ICG	d_H[a,c] [nm]	PDI[a,c]	zeta potential[b,c] [mV]	pH value
Solutol HS 15	11.9 ± 0.1	0.049 ± 0.008	−0.7 ± 1.0	6.6
Cremophor RH 40	13.6 ± 0.2	0.102 ± 0.007	−2.2 ± 0.8	6.8
Tween 80	8.4 ± 0.04	0.138 ± 0.004	−2.6 ± 0.8	6.7
sucrose ester L 1695	7.0 ± 0.05	0.245 ± 0.006	−7.6 ± 1.7	6.8

[a] Intensity-weighted mean hydrodynamic diameter and polydispersity index were determined by dynamic light scattering (DLS). Each measurement consisted of 30 runs, and the calculation mode used was CONTIN. [b] Zeta potential analysis was carried out using laser Doppler anemometry (LDA) with 30 runs per measurement. [c] The values represent the mean ± SD based on three individual measurements performed in triplicate.

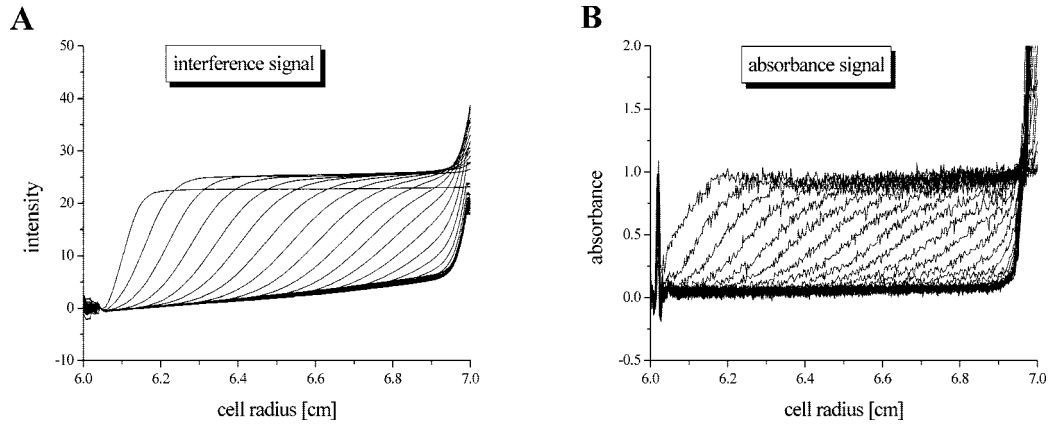

Figure 5. Sedimentation velocity experiments with ICG (10 mg/L) in Solutol HS 15 micelles (100 mg/mL). Investigations were performed at 45,000 rpm and interference signal (A) and absorbance signal (B) were scanned at 675 and 720 nm, respectively. Both diagrams had the same concentration profiles, indicating encapsulation of ICG within the micelles. Data shown are every fourth scan measured.

Table 4. Encapsulation Efficiency (EE) of ICG within Nonionic Micellar Systems

ICG formulation	residual absorbance[a] [OD]	residual ICG concn[b] [mg/L]	EE[c] [%]
Solutol HS 15	0.051	0.524	95
Cremophor RH 40	0.056	0.563	94
Tween 80	0.108	0.963	90
Pluronic F 68	0.485	3.867	61
sucrose ester L 1695	0.044	0.470	95

[a] The residual absorbance of free ICG in the supernatant was measured at run 131 within the range 6.3−6.9 cm cell radius. [b] Residual ICG concentration was determined by means of a calibration curve. [c] Encapsulation efficiency (EE) was calculated by the ratio of incorporated ICG to initial ICG concentration (10 mg/L).

were investigated: Solutol HS 15, Cremophor RH 40, sucrose ester L 1695, Tween 80 and Pluronic F 68.

The absorbance profiles before and after sedimentation indicated that ICG was nearly completely embedded in sucrose ester L 1695, Solutol HS 15, Cremophor RH 40 and Tween 80 micelles (90−95%). Pluronic F 68 micelles had an encapsulation efficiency of only 61% (Table 4).

Aqueous Stability. The aqueous stability of 5 mg/L ICG in specific nonionic surfactant systems stored in the dark was investigated at 4 and 25 °C over a period of 4 weeks. The various surfactant systems were found to have varying stabilizing capabilities. Storage of ICG in Solutol HS 15 or Cremophor RH 40 at 4 °C revealed no significant diminution of absorbance over the entire 4 week period (Figure 6A). Even at a higher temperature (25 °C), the absorbance value remained within the required range of 90−100% initial absorbance (95% and 91%, respectively). ICG in Tween 80 and Pluronic F 68 was less stable when stored at 25 °C (Figure 6B). The rapid degradation of free ICG in water was accelerated at higher temperatures in accordance with the literature.[13]

Hemolytic Activity. Hemolytic activity of ICG and micelles was determined by incubating red blood cells for 1 h with increasing concentrations of dye and surfactant. Nonisotonized, free ICG in water caused approximately 40% hemolysis at all investigated concentrations (Figure 7). Adjusting the osmolarity to physiological conditions using glucose resulted in a lowering of hemolytic activity, especially at low ICG concentrations. However, at a concentration of 5×10^3 mg/L ICG, hemolytic activity was still observed to be high with approximately 27% cell death.

Hemolytic activity of micelles was determined to be marginal at low concentrations (0.05 mg/mL surfactant). In sucrose ester L 1695, glycocholic acid and mixed micelles with sucrose ester S 1170, hemolysis increased to 80−100% cell death with increasing concentrations (Figure 8). In contrast to this, Solutol HS 15, Cremophor RH 40 and mixed micelles with Lipoid S 100 showed very low hemolytic activity (under 10%). Encapsulation of 500 mg/L ICG within 10 mg/mL Solutol HS 15 significantly reduced the toxic effects compared to free ICG in water (Figure 9).

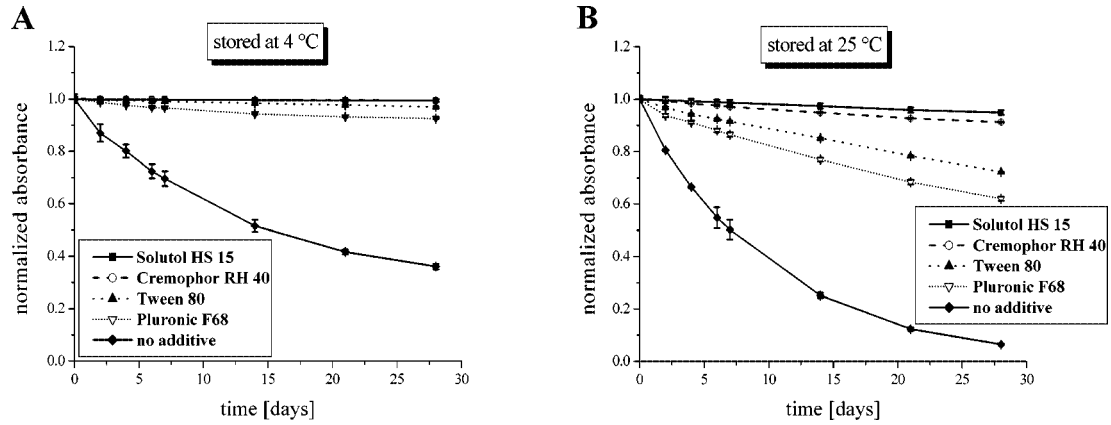

Figure 6. Aqueous stability of ICG (5 mg/L) in different nonionic surfactant systems (5% w/v) stored in the dark at 4 °C (A) and 25 °C (B). The maximum absorbance of each formulation was measured with plain micelles as reference, and the values were normalized for the initial absorbance. All micellar systems offered a higher stability for ICG in aqueous solution than free aqueous ICG. The initial absorbance value of ICG within Solutol HS 15 and Cremophor RH 40 micelles remained stable over a 4-week period when stored at 4 and 25 °C. Data represent means of six experiments ± SD (depicted as bars). When no bar is visible, the SD was smaller than the symbol.

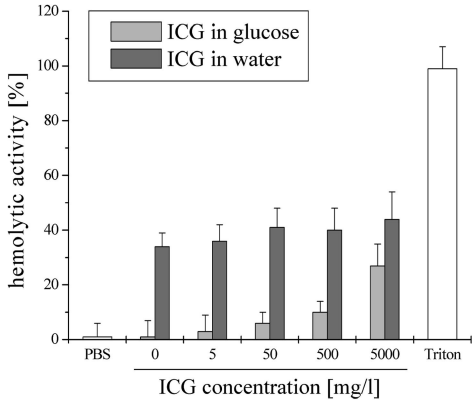

Figure 7. Hemolytic activity of free ICG in water and in glucose (55 mg/mL) Free ICG in water caused hemolysis up to 40% cell death whereas substitution with isoosmotic glucose solution reduced hemolysis.

Discussion. Aggregation in aqueous solution is a well-known phenomenon for various cyanine dyes.[8,44] The amphiphilic character of ICG leads to self-organization into highly ordered aggregates predominantly caused by van der Waals forces and hydrophobic interactions.[2,9,10,45] The dye−dye interactions have adverse effects on the optical properties, such as lower quantum yields due to self-quenching.[7] Spectral changes can be an indication of the formation of these aggregates. With increasing ICG concentration, the maximum in the absorption spectrum decreases, whereas the shoulder becomes more pronounced at $\lambda = 700$ nm, indicating the formation of dimers.[8] At even higher dye concentrations, a new absorbance maximum is eventually observed at $\lambda = 900$ nm, indicating the formation of larger aggregates, the J-aggregates.[9,44] To visualize the structure and morphology of the aggregates on a mesoscopic scale, cryogenic transmission electron microscopy (cryo-TEM) can be applied. This technique, which is well-known in biology, allows the characterization of water-soluble structures in their natural environment. The formation of J-aggregates in 10^3 mg/L aqueous ICG solution was detected after 2 weeks by means of the new maximum in the absorbance spectrum (Figure 1). At this time, the cryo-TEM image showed spherical aggregates, which turned into a large planar structure after a further 2 weeks (Figure 2). The sheet thickness of approximately 3.6 nm corresponds to the width of a typical biological lipid bilayer (3.6−4.3 nm).[46−48] This statement confirms the assumption of a bilayer formation of ICG molecules. The formation of threadlike aggregates reported in the literature could appear as an intermediate level between the spherical and sheet structure.[10] Kirstein and Daehne reviewed typical properties of amphiphilic cyanine dyes with the main focus on the relationship between structure and optical properties.[44]The investigated dyes had comparable structures to ICG. The bilayer structure of the ICG aggregates, found in the present study, corresponds well with the single narrow red-shifted absorption band in the absorption spectrum, as reported in the literature.[39,49]

Interaction of the amphiphilic ICG with micellar structures can reduce the formation of aggregates, especially the formation of dimers. The dye molecules incorporate into the interface of the surfactant micelle, leading to a partitioning

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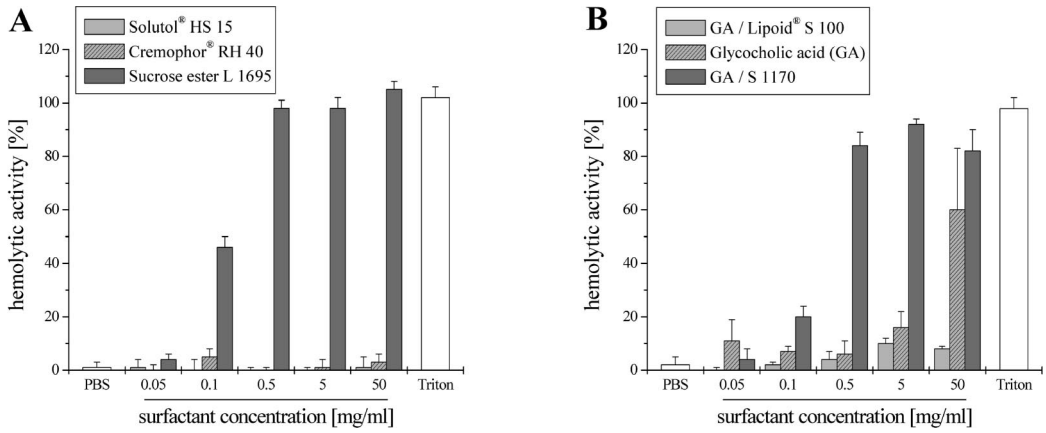

Figure 8. Hemolytic activity of different micellar systems. Human red blood cells were incubated at 37 °C for 1 h with different surfactants at various concentrations. After centrifugation, the percentage of free hemoglobin in the supernatant was determined photometrically at 540 nm. Triton X-100 served as positive control, PBS buffer as negative control. Only Solutol HS 15 and Cremophor RH 40 showed marginal hemolytic activity even at high concentrations.

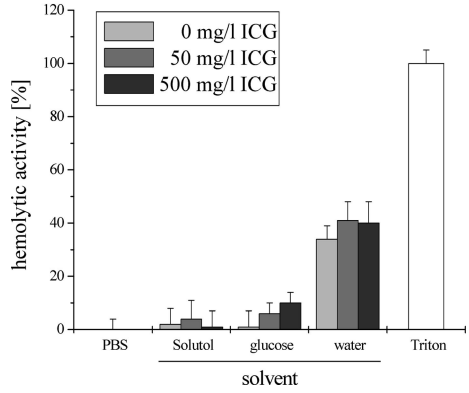

Figure 9. Hemolytic activity of free ICG in water and glucose (55 mg/mL) and encapsulated ICG within Solutol HS 15 micelles (10 mg/mL). ICG within Solutol HS 15 micelles and ICG within glucose displayed lower hemolytic activity than free aqueous ICG.

of ICG.[18] Thus, the optical properties of the ICG change. This results in higher fluorescence intensities, wavelength shifts and higher quantum yields. It can be concluded from Figure 3 that ICG, incorporated within micelles, has a lower aggregation tendency than free ICG, mainly at high concentrations.

A bathochrome wavelength shift as well as an increased quantum yield was observed for all ICG-encapsulated surfactant micelles which were tested. They all displayed a peak maximum shift of about 20−30 nm compared to the spectrum of ICG in water, except for ICG within Pluronic F 68 micelles. This finding may be attributed to the higher hydrophilicity of this polymer compared to other surfactants. The hydrophilic−lipophilic balance (HLB) value of Pluronic F 68 is 29,[50,51] which indicates a high proportion of hydrophilic groups within the molecule. The HLB value for nonionic surfactants whose only hydrophilic moiety is polyoxyethylene according to Griffin is described as follows:

$$\mathrm{HLB} = 20M_\mathrm{h}/M$$

where M_h is the molecular mass of the hydrophilic portion and M is the molecular mass of the whole molecule. The HLB value can exhibit values from 0 to 20. An HLB value of 0 corresponds to a completely hydrophobic molecule whereas a value of 20 corresponds to a molecule made up completely of hydrophilic components.[52] This HLB scale was expanded by Davies for more complex surfactants, thus, they can achieve higher numerical values than 20. The other nonionic surfactants are more lipophilic, with HLB values of approximately 15. A similar trend could be found for the amount of the quantum yield enhancement. ICG within nonionic surfactants, other than Pluronic F 68, showed an approximately 3-fold increase in the quantum yield compared to the free dye. In contrast, ICG within Pluronic F 68 micelles, showed only a marginal increase (Table 1). Encapsulation within ionic or mixed micelles resulted in an approximately 2-fold increase.

Most of the prepared micelles had particle sizes within the designated range of 10−100 nm. These small sizes allow the micelles to evade the RES because uptake is conspicuously lowered for particles of less than about 100 nm.[29−31,34] Presumably, the micelles will circulate in the blood for prolonged periods. The polydispersity varied from monodisperse to a broad distribution. The accurate determination of an average diameter of glycocholic acid, Pluronic F 68 and glycocholic acid/Lipoid S 100 micelles using dynamic

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light scattering was handicapped by their high polydispersity (PDI > 0.3). Further investigations and application of a multiple narrow mode for residual calculation of the autocorrelation function also failed. These difficulties confirm those found in previously published studies.[53−55]

The addition of ICG to selected plain micelles did not influence the particle size diameter, distribution or zeta potential. This may be attributed to the integration of the dye into the core of loose surfactant micelles with low packing density.[43,54]

ICG was nearly completely encapsulated within nonionic surfactants (90−95%), apart from Pluronic F 68 micelles (61%) (Table 4). This significant difference again relates to the high hydrophilicity of Pluronic F 68. Since ICG monosodium salt is an amphiphilic molecule with good water solubility ($c_s = 3.5 \times 10^4$ mg/L), it can be assumed that the surfactants used need to provide enough hydrophobicity to achieve sufficient hydrophobic interaction between the lipophilic parts of the dye and the lipophilic micelle core. Rodriguez et al. tried to obviate this problem by using the more lipophilic ICG tetrabutylammonium iodide salt, expecting an increased micelle loading.[25] Compared to the encapsulation efficiencies of 90−95% reported in this study, they reported a loading efficiency of only 87%. The present study shows that the surfactants used, with HLB values of about 15, are well able to encapsulate ICG, even the hydrophilic monosodium salt.

Various previous studies investigated the aqueous stability of ICG encapsulated in nanosized carriers.[23−25,27,56] All of them were able to show that aqueous stability was increased, whereas most of them only covered a period of 3−5 days. Rodriguez et al. obtained aqueous stability of ICG-encapsulated micelles over 2 weeks at room temperature. Higher temperatures (37 °C) decreased the fluorescence emission to 63% after 2 weeks.[25] The investigations in the present study exhibited no significant loss in absorbance for ICG within nonionic micelles over a 4 week period with storage at 4 °C. Encapsulation within Solutol HS 15 micelles preserved the initial absorbance of ICG even at room temperature after 4 weeks, which demonstrates the superior stabilization (Figure 6). In contrast, the absorbance of free aqueous ICG diminished to a poor 5% of the initial value in the meantime. It is quite obvious that the micelles protect the ICG from degradation, presumably by isolating the dye from the aqueous environment. Consequently, the hydrolyzed fragile structures of ICG seem to remain in the core of the micelles.

Surfactants used for parenteral application may induce hemolysis, a rupture of red blood cells which accompanies the release of hemoglobin. The reasons for this are osmotic effects due to an increased membrane permeability or solubilization of membrane lipids and proteins caused by the use of surfactants.[57] Incubation of red blood cells with the various surfactants can give a first impression of the toxicity of the micellar formulations *in vivo*. To compare the micellar systems to the currently used ICG formulation, different concentrations of free dye in water were investigated in addition to the surfactant systems. All of these aqueous dye solutions exhibited very low osmolarity (5×10^3 mg/L ICG in water = 20 mOsm/kg). Several previous publications have already described the toxic effects of ICG in relation to osmolarity.[58−60] Namely, an incubation time above 5 min with slightly hypo-osmolar solution (282 mOsm/kg) reduced cell viability of ARPE-19 cells *in vitro*. This effect was found to depend on osmolarity rather than on dye concentration.[60] Stalmans et al. also demonstrated that toxic effects of ICG relate to hypo-osmolarity of the solvent, by comparing ICG in water to infracyanine green in 50 mg/mL glucose.[58] Similar observations were found for the hemolytic investigation in the present study. ICG dissolved in water, the form in which it is currently clinically used, caused hemolysis at all shown concentrations, in the same way as pure water. The use of 55 mg/mL glucose as a solvent significantly reduced the hemolytic activity as shown in Figure 7. However, the clinically relevant concentration of 5×10^3 mg/L ICG still induces approximately 30% cell death even in isoosmolar glucose solution. The amphiphilic character of ICG itself could be an explanation for this effect at high dye concentrations. These results demonstrate a high sensitivity of the hemolytic assay. Toxicity of ICG *in vivo* might be less due to the plasma protein binding.

In general, nonionic surfactants exhibit lower negative side effects than ionic surfactants.[61] Previous studies of sugar-

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based surfactants demonstrated high hemolytic activity compared to commercially available surfactants such as Solutol HS 15, Brij and Triton X-100.[62,63] These findings confirm the results found in the present study. The two sugar ester formulations which were studied exhibited the highest hemolytic activity, followed by the ionic surfactant glycocholic acid (Figure 8). By preparing mixed micelles of glycocholic acid with Lipoid S 100 the toxic effect could be minimized. This protective nature of phosphatidylcholine has already been described in several publications and can be explained by a lower solubilizing capacity of the mixed micelles for membrane components.[64,65] Compared to this, Solutol HS 15 and Cremophor RH 40 micelles showed hardly any hemolytic activity (Figure 8A). Encapsulation of 500 mg/L ICG within 10 mg/mL Solutol HS 15 demonstrated that the hemolytic effect of free ICG in water was significantly reduced (Figure 9).

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These results show clearly that surfactant micelles are useful tools with which to overcome the drawbacks of ICG. Further studies of *in vitro* toxicity, micelle stability and plasma half-life are currently ongoing.

Conclusion

Indocyanine green was encapsulated via a simple dissolution method within biologically compatible surfactant micelles. Characterization of the systems demonstrated that the most suitable encapsulation for ICG was within Solutol HS 15 micelles. Dimerization of the cyanine dye was reduced compared to free ICG, leading to less self-quenching and hence a significantly higher quantum yield. Absorbance and fluorescence spectra were bathochrome-shifted by 20 nm. The micelles were monodisperse with an average diameter of 12 nm and a zeta potential close to zero. ICG within Solutol HS 15 micelles was stable over a four-week period when stored at 4 and 25 °C. The hemolytic activity of free ICG in water was significantly reduced by incorporation into the micelles.

Solutol HS 15 micelles are potential nanocarriers for ICG that improve the optical properties of the dye and provide for high aqueous stability and low hemolytic activity. By means of incorporation into the micelles, ICG can be prevented from binding to plasma proteins which could increase the half-life *in vivo*.

Acknowledgment. The authors thank Dr. C. Böttcher from the Electron Microscopy Research Center at Freie Universität Berlin for the Cryo-TEM investigations.

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