Studies on biocompatible nanocapsules formed in microemulsion templated processes

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Phase diagrams of the pseudoternary system: N-alkyl-N-methylgluconamines/isobutanol/isoctane/water have been determined. Size and morphology of organic/water microemulsion droplets (optically isotropic, low viscosity one-phase systems) have been characterized by dynamic light scattering (DLS) to select systems suitable to prepare by interfacial polymerization poly(ethyl-2-cyanoacrylate) nanocapsules, the latter analyzed by scanning electron microscopy (SEM). The diameter of the nanocapsules (ranging from 300 to 1550 nm) was found to be dependent on the the monomer mass used in the polymerization process. Investigation of those nanoparticles as a cyanine IR-768 carrier, for enhancing the photosensitizer selective delivery to tumor cells, has been performed. Incubation of MCF-7 Dox cells with cyanine dye IR-768 (Indoc5), free or encapsulated in nanoparticles, allowed the cellular accumulation of the photosensitizer. Our preliminary experiments indicate that higher doses of encapsulated cyanine improved the cytotoxic effect.

Key words: nanocapsule; microemulsion; alkylecyanoacrylate; IR-768 perchlorate; MCF-7 Dox; cytotoxicity

1. Introduction

Oil-cored biodegradable nanocapsules suitable for the hydrophobic drugs encapsulation can be prepared by interfacial polymerization of oil-in-water (o/w) emulsions or microemulsions [1–4]. The use of the latter as templates for the preparation of nanocapsules by interfacial polymerization offers advantages over the use of size-reduced kinetically stabilized emulsions. Furthermore, if biocompatible oils and surfactants are used for the formation of microemulsions, the necessity of isolating the nanocapsules from the reaction matrix following polymerization is omitted [5]. Nanocapsules can therefore be prepared in situ in a microemulsion matrix.

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The main purpose of the present study was to find and characterize a microemulsion single phase region in pseudoternary phase systems with a potential application in drug delivery, especially as templates for synthesis of drug loaded nanocapsules. The o/w microemulsion isotropic area was found in the ternary systems: N-alkyl-N-methylglucamine/isobutanol/isooctane/water, and utilized in the template-directed polymerization of reactive ethyl 2-cyanoacrylate (ECA) monomer, solubilized in the o/w droplet interfacial area. The findings described in the paper have not been reported so far.

In biological studies, we applied the particles to the delivery of some photosensitizers to tumor cells in photodynamic therapy (PDT) – an alternative modality of cancer treatment. It has to be emphasized that the photosensitizer which is absorbed by all cells and selectively retained by malignant tissue, after light exposure is promoted to an excited state and induces local release of cytotoxic reactive oxygen species (ROS). Depending on the experimental conditions and cell sensitivity, the cytotoxic molecular species resulting from PDT may trigger cell apoptosis or induce necrosis [6]. In clinical PDT, the same side effects were observed as a result of the dark toxicity of photosensitizers towards normal tissues. Low dark toxicity is one of the important criteria for assessing the usefulness of photosensitizers [7]. In our experiments, we used cyanine IR-768 perchlorate in a free form and encapsulated in nanoparticles to determine their incorporation to the breast cancer MCF-7 Dox cells. The near-IR spectral region is particularly suitable for the PDT as it penetrates deeply into tissues without causing significant heating. It is selectively concentrated and retained in vitro and in vivo to greater extent in the mitochondria of carcinoma and melanoma cells than in normal cells [8].

2. Experimental

*Materials.* N-decanoyl and N-octanoyl-N-methylglucamine, isobutanol, ethyl 2-cyanoacrylate (ECA), chloroform, ethanol and the photosensitizer (Fig. 1): 2-[2-[3-[(1,3-dihydro-3,3-dimethyl-1-propyl-2H-indol-2-ylidene) – ethylidene]-2-phenoxy-1-cyclohexen-1-yl]ethenyl]-3,3-dimethyl-1-propylindolium perchlorate (IR-768) were purchased from Sigma Aldrich. Isooctane was obtained from Carl Roth KG Chemicals. Distilled water buffered to pH 7.4 with phosphate buffer was used in the experiments with ECA. Water used for all experiments was doubly distilled and purified in a Millipore (Bedford, MA) Milli-Q purification system.

![Fig. 1. Structure of the IR-768 photosensitizer](image-url)
Phase diagrams. The four component systems were described by pseudo-ternary phase diagrams at 25 °C. All samples were prepared at a 1:1 surfactant-to-cosurfactant weight ratio. Surfactant/sobutanol/isooctane mixtures were diluted with water, after each dilution the samples were vigorously shaken and left for 24 h to attain equilibrium. Visual observations and cross-polarizers were used to identify the systems.

Preparation of nanocapsules. Ethyl-2-cyanoacrylate monomer (5, 10, 20, 30 μl) dissolved in of chloroform (20–90 μl) was slowly added to 2 ml of selected microemulsion template (or microemulsion containing IR-768). Polymerization was performed at 4 °C and the system was stirred for at least 4 h. Nanocapsules were collected by centrifugation for 15 min at 25°C (Unipan 310 rotor). A Metertech SP8001 spectrophotometer with 1 cm path length quartz thermostated cell was used to determine the amount of photosensitizer solubilized in microemulsions or nanocapsules. IR-768 was detected at 768 nm. The obtained nanospheres were dispersed in the PBS solution.

Characterization of nanocapsules. The particle size and distribution of the PECA nanocapsules was determined by the dynamic light scattering (DLS, Zetananosizer Nano series ZS, Malvern Instruments Ltd.). Residual oil and surfactant were removed by repeated washing in ethanol, centrifugation (15 min at 25 °C) and then the dry nanocapsules were dispersed in ethanol. The external structure of nanocapsules was visualized by SEM (Jeol, JSM-5800 LV). The nanocapsules were coated with carbon before microscopic observations and viewed at an accelerating voltage of 22 kV.

Cell culture. Human breast cancer MCF-7 Dox cells (doxorubicin resistant cell line) were grown at 37 °C in a humidified atmosphere (95% air and 5% CO2), DMEM (Dulbecco’s Modified Eagle medium) supplemented with phenol red, 3% L-glutamine, penicillin (100 U/ml), gentamycin (100μg/ml) and 10% foetal calf serum. Fresh medium was supplied every 3 days.

Photosensitizer and photosensitization. The cyanine dye IR-768 (Indoc5) was prepared as 1.4 mM stock solution by dissolving 1 mg of green powder in 1 ml of 0.1% DMSO/PBS, pH 7.4. Photosensitization experiments were performed with MCF-7 cells (5×10⁴ cells/well) incubated in a 96-well microplate (Nunc, Denmark). Cyanine was then added from the stock solution to attain final concentrations ranging from 7 to 56 μM. Equivalent amounts of 0.1% DMSO were added to other wells with cells that served as control. After 24 h incubation, the cells were photoirradiated (except the dark controls). The light source was a lamp (OPTEL, Opole, Poland) with polarized light and yellow filter (λ = 620 nm). The nominal output energy (continuous wave) was 0.13 mW/cm². Then the cells were incubated at 37 °C, 5% CO₂ – air atmosphere during 24 h in a drug-free medium. Experiments with encapsulated cyanine were performed in the same manner.

Cytotoxicity assay. Cell viability after irradiation was determined by the (MTT) 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide assay [9]. The absorbance of degraded MTT at 570 nm was measured using an ELISA reader (Labsystems Multi-Scan MS, Finland). All experiments reported herein included several controls.
Fluorescence microscopy. Microcultures derived from the culture dishes were conducted on 8-well glass slides. Subsequently, the cells were incubated with 47 μM IR-768 (encapsulated and free dye) for 24 h at 37 °C. After incubation, the cells were fixed in 4% buffered formalin, washed with PBS and examined under a fluorescence microscope (Olympus BX51) using a yellow filter Y3Y3 (λex. = 612 nm).

3. Results and discussion

Odourless, nontoxic, non-irritant to eyes and skin, biodegradable surfactants, N-alkyl-N-methylgluconamines, appeared to be good candidates for stabilizing both o/w and water-in-oil (w/o) microemulsions with potential applications in various fields, for example as templates for biodegradable nanocapsules or as original drug carrier systems for transdermal delivery.

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>n</th>
<th>Abbreviation</th>
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| \[
\begin{array}{c}
\text{HO} \\
\text{OH} \\
\text{OH} \\
\text{CH}_3 \\
\text{OH} \\
\text{OH} \\
\text{N} \\
\text{C} \\
\text{C}_n\text{H}_{2n+1} \\
\text{O}
\end{array}
\] | 8  | C_8-MEGA     |
| \[
\begin{array}{c}
\text{HO} \\
\text{OH} \\
\text{OH} \\
\text{N} \\
\text{C} \\
\text{C}_{10}\text{H}_{22} \\
\text{O}
\end{array}
\] | 10 | C_{10}-MEGA  |

Fig. 2. Structures of N-alkyl-N-methylgluconamines, C_n-MEGA

The selected N-alkyl-N-methylgluconamines (Fig. 2) when added to a three-component system (cosurfactant/isooctane/water) make it possible to obtain either w/o or o/w and bicontinuous (bc) microemulsions. Figure 3 shows the pseudoternary phase triangles for the systems with N-alkyl-N-methylgluconamines of different chain lengths. The black areas represent microemulsion regions, the white ones representing anisotropic, two-phase regions. The length of the surfactant alkyl chain strongly influenced on the shape and extent of the microemulsion area; the largest microemulsion area was obtained for the system containing the surfactant with the decyl hydrophobic chain (C_{10}-MEGA). For further studies some microemulsion systems based on the constructed pseudoternary phase diagrams have been selected and prepared.

The sizes and size distributions of droplets in microemulsions (at some compositions) and in nanocapsules were characterized by the DLS method. The corresponding diameters of microemulsion droplets (D) and polydispersity index PdI are given in Table 1. These systems were subject to interfacial polymerization and biological studies. The sizes of microemulsion droplets ranged from 6.4 to 8.1 nm and from 9.6 to 13.2 nm for C_8MEGA and C_{10}MEGA surfactants, respectively, making them good candidates for template-based reactions.

It can also be seen that the micelle size increases with the content of isooctane. In the case of formed nanocapsules, the size ranges from around 300 to 1550 nm. Probably a decrease in the mass of monomer available per interfacial area unit causes that...
Biocompatible nanocapsules formed in microemulsion templated processes

nanocapsules have thinner polymer walls and hence smaller sizes. Nanocapsules of the order of 1000 nm in diameter have not been applied to biological studies. An increase in the particle size occurs with increasing the monomer concentration used in the polymerization process (Table 2). Furthermore, the diameters of empty nanocapsules were slightly smaller than those loaded with the photosensitizer. All nanocapsules were spherical as observed by SEM (Fig. 4a, b).

![Phase diagrams of the N-alkyl-N-methylgluconamide C₈-MEGA/isobutanol/isooctane/water ternary systems at 25 °C: a) C₈-MEGA, b) C₁₀-MEGA. Black areas represent the microemulsion monophases, white – two phase regions; W – water, I – isooctane, Cos – isobutanol (cosurfactant)](image)

**Table 1. Diameter (D) and polydispersity index (PDI) of the microemulsions droplets obtained by the DLS method for C₈-MEGA/cosurfactant/isooctane/water systems**

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Weight fraction [%]</th>
<th>D [nm]</th>
<th>PDI</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Surfactant/isobutanol (1:1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oil</td>
<td>Water</td>
</tr>
<tr>
<td>C₈-MEGA</td>
<td>35</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>10</td>
<td>55</td>
</tr>
<tr>
<td>C₁₀-MEGA</td>
<td>25</td>
<td>5</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3</td>
<td>82</td>
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The efficiency of IR-768 entrapment within poly(ethyl-2-cyanoacrylate) o/w microemulsions-templated nanocapsules was influenced by the mass of monomer used in the polymerization (Table 2). The encapsulation of the examined photosensitizer with-
in PECA nanocapsules increased with increasing the monomer concentration from 32.9–39.0% for 2.5×10^{-3} \mu l/ml to 88.5–78.7% for 12.5×10^{-3} \mu l/ml.

**Table 2. Characterization of empty and loaded nanocapsules**

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>Concentration of monomer [10^{-3} \mu l/ml]</th>
<th>Nanocapsules</th>
<th>Encapsulation [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without IR 768</td>
<td>With IR 768</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D [nm]</td>
<td>PDI</td>
</tr>
<tr>
<td>C_{8-}MEGA</td>
<td>2.5</td>
<td>207</td>
<td>0.740</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>390</td>
<td>0.475</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>962</td>
<td>0.611</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>1368</td>
<td>0.754</td>
</tr>
<tr>
<td>C_{10-}MEGA</td>
<td>2.5</td>
<td>367</td>
<td>0.298</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>647</td>
<td>0.708</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1310</td>
<td>0.454</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>2660</td>
<td>0.250</td>
</tr>
</tbody>
</table>

Fig. 4. Scanning electron micrographs of nanocapsules:

a) in the o/w microemulsion template, b) from the sample pallet

MCF-7 cells are remarkably resistant to various treatments including chemotherapeutic cisplatin and some types of photosensitizers used in PDT [10]. Previous studies demonstrated the effect of indocyanine green ICG775 on the survival of MCF-7 cells [11]. In our experiments, we established whether cyanine IR-768 photosensitizer (Fig. 1) can be delivered to cancer cells in a free form or via nanoparticles. The concentration of IR-768 in nanocapsules chosen for cytotoxicity experiments was 0.517 mg/ml. In the fluorescence microscopy, the free IR-768 cyanine penetration in the cytoplasm of the MCF-7 Dox cell line after 24 h incubation was observed (Fig. 5a). The fluorescence of the dye molecules incorporated into nanocapsule particles was located in cytoplasm as well (Fig. 5b). A weak intensity of the fluorescence (Fig. 5b) was probably caused by the covering effect of the nanocapsule wall.
Biocompatible nanocapsules formed in microemulsion templated processes

Determination of cell survival by MTT colorimetric assay reflect metabolic conditions in mitochondria compartment and concerns mitochondrial dehydrogenase activities [12]. The viability of MCF-7 Dox cells upon photoirradiation in the presence of free IR-768 or encapsulated IR-768 were evaluated by the MTT assay as a function of dye concentrations (Fig. 6). Encapsulated IR-768 showed significant lower cytotoxicity than free cyanine. Evidently, nanocapsule prevents cells from the contact with the dye particles, being itself less toxic for the cells. However, cytotoxic effect of IR-768 loaded nanoparticles was increased under irradiation. Microemulsions used for the delivery of IR-768 into MCF-7 Dox cells caused a strong cytotoxic effect in the dark conditions (data not shown). For these reasons they were not useful for further PDT studies.
Our preliminary in vitro studies in MCF-7 Dox cell lines showed that nanocapsule particles may be used as carriers of a photosensitizer. IR-768 in a nanocapsule may represent a useful formulation of the photosensitizer for practical PDT. Free IR-768 caused high cytotoxicity effect independently of light treatment. In spite of these effects, it cannot be used in a free form because IR-768 is poorly soluble in polar solvents. The problem of a weak solubility of IR-768 in polar environment could be solved by incorporating the dye into biocompatible nanocapsules. These carriers penetrate tumor cells but a higher concentration of IR-768 should be used to obtain more effective cytotoxicity. In future experiments, we will use nanoparticles with thin walls to obtain a better cytotoxic effect after irradiation of tumor cells. A very important problem is selective targeting of a drug carrier to subcellular mitochondrial compartment for induced apoptosis. Apoptosis has been shown to be a rapid and dominant form of cell death following photodynamic therapy. Tumor cell death by necrosis is an undesirable effect because of the inflammatory state generated in the process [13]. The construction of nanocapsules with specific tumor targets should improve the effective treatment and could be safer for normal cells. Future research will include analysis of metabolic state of cells exposed to photosensitization after using nanocapsule particles as carriers of cyanine dyes.

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References


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