Biodegradable Nanospheres Containing Phthalocyanines and Naphthalocyanines for Targeted Photodynamic Tumor Therapy

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Preparation methods of cyanoacrylic nanocapsules or nanoparticles containing phthalocyanines and naphthalocyanines are described. Nanocapsules were obtained by interfacial polymerization in an oil-in-water emulsion. Drug encapsulation efficiency depended upon drug concentration, ethanol concentration, and phthalocyanine sulfonation degree and reached 100% in some cases. Nanocapsules size ranged from 150 to 250 nm and varied with phthalocyanine sulfonation degree and pH of the aqueous phase. Nanoparticles were prepared by the addition of monomer to an aqueous phase containing hydrophilic phthalocyanine derivatives. Depending upon the pH, sizes ranged from 10 to 380 nm. Drug binding was between 75 and 80%. These new preparations could prove useful in the photodynamic treatment of tumors.

KEY WORDS: polymeric nanospheres; alkylcyanoacrylates; phthalocyanines; naphthalocyanines; photodynamic therapy; drug targeting.

INTRODUCTION

In photodynamic therapy (PDT) of neoplasms (1), photosensitizer is administered to the patient followed, after 24–48 hr, by exposure of the diseased area to red light, usually delivered by an argon-dye laser. Advantages of PDT over conventional cancer therapy include selectivity and low systemic toxicity, allowing its application even to areas which have already received maximal doses of radiotherapy. The photosensitizer preparation that is almost exclusively used in clinical trials of PDT consists of a mixture of hematoporphyrin derivatives (Photofrin II) which exhibit a weak absorption maximum at 630 nm. The procedure could be improved with the use of photosensitizers that strongly absorb red light above 650 nm, where tissue exhibits optimal transparency and where the development of reliable, inexpensive diode lasers is feasible. To this end several new classes of potential sensitizers for PDT have been developed, such as phthalocyanines (Pc) and naphthalocyanines (NPc) (2). They resemble naturally occurring porphyrins in many aspects (Fig. 1) but have the advantage over porphyrins in that they absorb strongly above 650 nm, i.e., in the clinically useful red region of the spectrum. The cytotoxic species believed to be responsible for the tumor response is singlet oxygen, formed via a type II energy transfer process between the triplet M-Pc and ground-state molecular oxygen (2).

Nonsubstituted M-Pc's are insoluble in aqueous media, and most *in vivo* studies have been done with the water-soluble sulfonated analogues, M-PcS_n, where n depicts the degree of sulfonation. Both Al-PcS_{1-4} and Zn-PcS_{1-4} have been shown to possess photodynamic activity in experimental mouse tumors comparable or superior to that observed with Photofrin II. Nonsubstituted Zn-Pc, which is more readily available as a single, pure compound as the variably sulfonated analogues, has been incorporated into unilamellar liposomes for *in vivo* administration and shown to induce a good PDT response (4). However, only a limited fraction of the dye can effectively be incorporated into liposomes. Moreover, liposomes are hampered by their relative instability upon storage and in biological fluids (5). Finally, a standard manufacturing process of sterile pyrogen-free liposomes devoid of unencapsulated drug has still to be developed.

As an alternative to liposomes, cyanoacrylic nanospheres were proposed several years ago (6). Their main advantages lie in biodegradability (7), a high drug absorption capacity, and a manufacturing process allowing the preparation of stable, sterile, and pyrogen-free nanospheres with hardly any free drug left (8). In this work we have studied the preparation method of plain poly-isobutyl (IBCA)- or ethylbutylcyanoacrylate (EBCA) nanoparticles linked to metallophthalocyanines and their sulfonated derivatives. In addition to these plain polymeric nanospheres, phthalocyanines were also linked to EBCA nanocapsules consisting of a lipidic core surrounded by a polymeric wall (12).

MATERIALS AND METHODS

Phthalocyanine Derivatives

In order to obtain pure zinc 4,4′,5″,5″-tetrasulfophthalocyanine (Zn-PcS_4), the sodium salt of 4-sulfophtalic acid was condensed with $ZnSO_4$ in the presence of urea. The condensation reaction favors the formation of one of four possible constitutional isomers. Formation of a single product was confirmed by reverse-phase HPLC analysis (9,10).

In order to obtain mono- to tetrasulfonated derivatives, phthalocyanines complexed with $AlCl^{2+}$ and Zn^{2+} (Kodak, Rochester, NY) were sulfonated directly by treatment with fuming (30%) H_2SO_4 for 2.6 hr at 75°C (10,11). This procedure yields a mixture of mono-, di-, tri-, and tetrasulfonated Zn-PcS (Zn-PcS_{1-4}). Purification of these compounds was effected by medium-pressure chromatography on a C-18 reverse-phase column using a linear gradient of MeOH in phosphate buffer (10). The nonsulfonated silicon naphthalocyanine $Si(OR_3)_2$-NPc, with R-C_6H_{13} was prepared according to a published method (19). The AlCl-NPc was prepared in a similar manner via condensation of 2,3-dicyanonaphthaline and $AlCl_3$ (anh.) at 270–280°C for 1 hr.

Nanocapsule Preparation

Nanocapsules were prepared by the addition of 5 ml of

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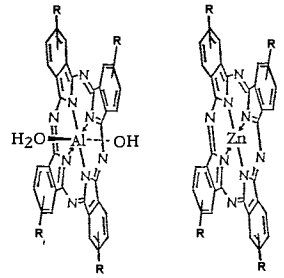

Fig. 1. Chemical structure of Al- and Zn-PcS. R = H or SO_3^-, depending on the degree of sulfonation.

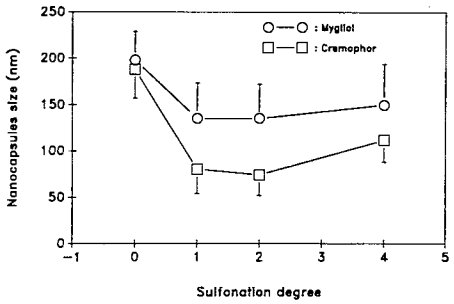

Fig. 2. Nanocapsule size as a function of the degree of sulfonation of Zn-PcS.

an organic phase containing Pc/Npc and monomer to an aqueous solution. Typically, the organic phase consisted of 2% mygliol 829 or cremophor, 0,9% IBCA or EBCA, 0.2–0.5% (w/v) M-Pc, M-PcS, or Npc in 5 ml of ethanol, while the aqueous phase was 10 ml of a 0.25% poloxamer 407 solution in 5 m*M* phosphate buffer at pH 7.0. Preparation methods have been described by Al-Khoury *et al.* (13) for IBCA and Chouinard *et al.* for EBCA (12). When the most hydrophylic metallophthalocyanines were used (M-PcS_4), they were dissolved in the aqueous phase.

Nanoparticle Preparation

Nanoparticles were prepared according to the technique of Couvreur *et al.* (14). IBCA or EBCA was added (10 mg/ml) to two different aqueous media (pH 2.5 and 7.0) containing 1% dextran or 5% DEAE-dextran, glucose, and 5 m*M* phosphate buffer. Polymerization was complete after 2 hr (IBCA) or 6 hr (EBCA), after which suspensions were neutralized when necessary using disodium phosphate. Because the drug had to be dissolved in an aqueous phase, only the tetrasulfonated Zn-PcS_4 was prepared in this manner. The initial concentration of Zn-PcS_4 was 0.453 mg/ml.

Phthalocyanine Derivative Binding Efficiency

Samples of the various batches of nanoparticles and nanocapsules were centrifuged at 100,000*g* for 1 hr. Drug concentration in the supernatant was determined by direct spectrophotometric analysis of the monomeric dye at 668 nm for Zn-PcS, 674 nm for Al-PcS, and 770 nm for $Si(OH_3)_2$-NPc.

Binding efficiency is given by

$$\% \text{ binding} = 100 \times \frac{\text{initial concentration} - \text{concentration in supernatant}}{\text{initial concentration}}$$

Nanosphere Characterization

Nanosphere size was analyzed by photon correlation spectroscopy using a laser beam scattered at 90° (N4SD Nano-Sizer, Coulter Electronics Ltd., U.K.).

Binding Stability

In order to check the stability of the binding of Zn-PcS_4 to nanocapsules, the preparations were centrifuged (55,000*g*, 2 hr) and resuspended in water five times consecutively with 24-hr intervals between each washing. Zn-PcS_4 was assayed by direct spectrophotometry at 668 nm in the supernatants and in the last pellet after dissolving the material in dimethylformamide.

RESULTS

Nanocapsule Size

The effect of different variables on EBCA-nanocapsule size was studied. As shown in Fig. 2, sulfonation of the phthalocyanine played a significant role in controlling particle size. More precisely, capsules prepared with unsulfonated M-Pc were larger than those prepared with sulfonated M-PcS, irrespective of the sulfonation degree. Polymerization of cyanoacrylates is initiated by anions and other neutrophils as shown in Fig. 3. In the organic phase, the presence of SO_2 inhibits anionic polymerization. Upon emul-

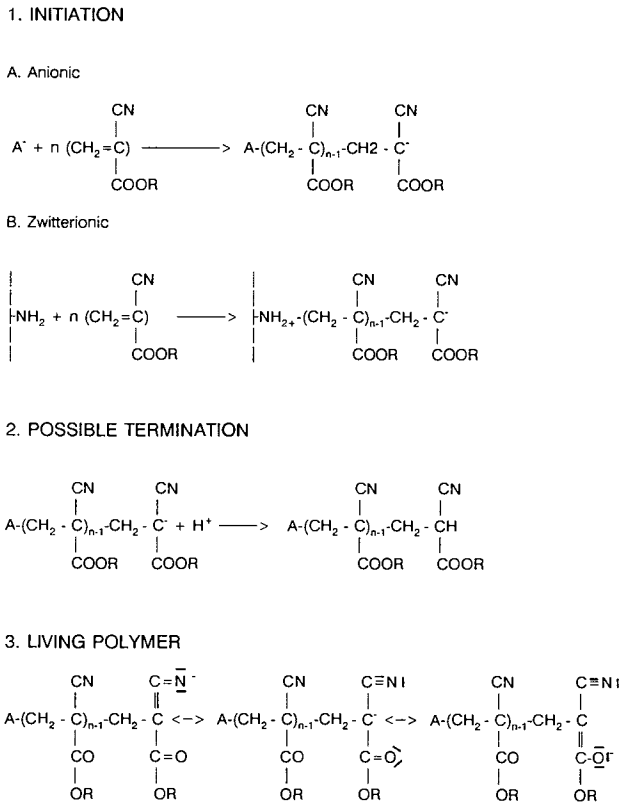

Fig. 3. Anionic and zwitterionic polymerization mechanism of alkylcyanoacrylate and canonic forms of the living polymer.

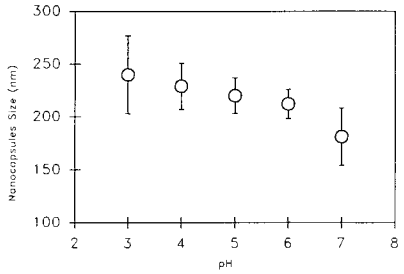

Fig. 4. Nanocapsule size as a function of aqueous phase pH.

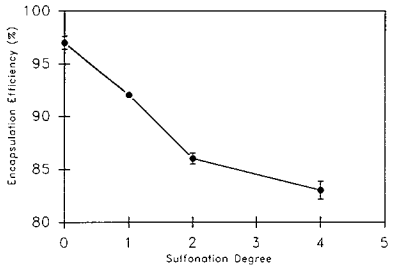

Fig. 5. Zn-PcS_{0-4} nanoencapsulation efficiency as a function of sulfonation degree.

sification, however, SO_2 diffuses in water and polymerization can take place (12). Sulfonates, being strong nucleophils, are likely to initiate the reaction, thereby developing covalent bonds with the polymer. The formation of this new chemical entity obviously influences polymerization kinetics and nanocapsule size, although the exact mechanism of this interaction remains unknown.

Effect of organic phase composition on nanocapsule size was also studied. When cremophor was used instead of mygliol, the size was approximately one-half. Similar sizes were obtained for emulsions prepared in the same manner without polymeric wall, indicating that nanocapsules size is greatly influenced by physicochemical characteristics of the organic phase. Monomer concentration (10 to 70 mg/ml in the initial oily phase) and drug concentration (0.3 to 4.6 m*M* in the final suspension) had hardly any effect on nanocapsule size. Increasing the OH^- concentration resulted in smaller sizes (Fig. 4). Hydroxyl ions are known to initiate the polymerization reaction (Fig. 3). Thus, the effect of sulfonate groups or hydroxyl ions seems to be that of a polymerization initiator, with higher initiator concentration resulting in a reduction of nanocapsule size.

The size of nanocapsules prepared with $Si(OR_3)_2$-Pc and $Si(OR_3)_2$-NPc were not significantly different, indicating that the presence of naphthalo groups had no effect on nanocapsules size (Table I). In this case also, a marked difference was noted between capsules prepared with cremophor and capsules prepared with mygliol. Using AlCl-NPc resulted in smaller nanocapsules, which is likely related to the absence of a second axial ligand as in the case of the $Si(OR_3)_2$-NPc.

Nanoencapsulation Efficiency

Sulfonation of Zn-Pc had a negative effect on encapsulation efficiency as can be seen in Fig. 5. Increasing the degree of sulfonation enhances the hydrophilicity of Zn-PcS. Consequently, in an oil-in-water emulsion, the fraction of the drug in the oily phase decreases with increases in degree of sulfonation, while the nanoencapsulation efficiency is reduced. This result is consistent with earlier reports (16) showing that nanoencapsulation efficiency is controlled by the partition coefficient of the drug.

Surprisingly, when Zn-PcS_4 was dissolved in the aqueous phase, 83% of the drug was bound to nanocapsules, whereas with AlCl-Pc no binding was achieved. It is possible that the sulfonate groups of Zn-PcS_4 could directly initiate cyanoacrylate polymerization and develop covalent links with the polymeric wall. In this case, it would be logical that AlCl-Pc, being devoid of sulfonates, was not able to initiate cyanoacrylate polymerization and bind to the polymeric wall. The development of covalent bonds between nucleophilic group-containing molecules (i.e., phenylbutazone) and alkylcyanoacrylates has been demonstrated (21). The binding stability assay showed that 85% of the initial dye remained in the capsules, while 17% leaked in the first supernatant corresponding to the fraction of the dye that was not linked to the polymer at the initial stage, whereas no dye was detected in the four subsequent supernatants. These results indicate that Zn-PcS_4 binds strongly to the polymer, resisting detachment in consecutive washings.

Finally, we observed that increasing the ethanol concentration had a positive effect on Zn-Pc encapsulation efficiency (Fig. 6). Since the drug is insoluble in pure ethanol, it is unlikely that the ethanol concentration significantly altered drug partition between the phases. However, higher ethanol concentrations reduced nanocapsules size and, consequently, increased the specific surface area of polymeric walls. If the drug encapsulation results from an interaction with the polymeric wall, the increased encapsulation efficiency appears logical. The same explanation may account for the gradual decrease in nanocapsule association percentage with increasing drug concentration (Fig. 7). Indeed, after

Table I. Characterization of Nanocapsules Containing Metallophthalocyanines

PC derivative	Solvent	Nanocapsule size (nm)	Nanoencapsulation (%)
NPc-$(OSiR)_2$	Mygliol	290 ± 47	100
NPc-$(OSiR)_2$	Cremophor	174 ± 41	100
NPc-$(OSiR)_2$	Acetone	184 ± 37	100
NPc-AlCl	Mygliol	162 ± 31	100
NPc-AlCl	Cremophor	117 ± 20	100

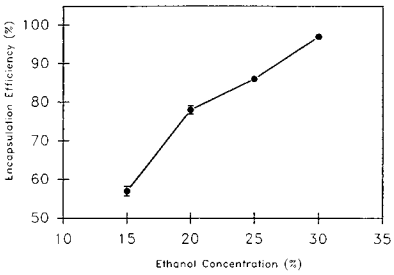

Fig. 6. Zn-Pc nanoencapsulation efficiency as a function of ethanol concentration.

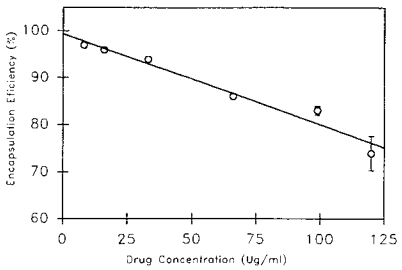

Fig. 7. Zn-Pc nanoencapsulation efficiency as a function of drug concentration.

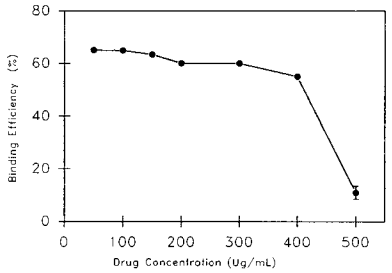

Fig. 8. Binding efficiency of Zn-PcS_4 to nanoparticles as a function of drug concentration.

saturation of the binding sites drug in excess remains in the free form.

Using naphthalocyanines, all nanocapsule preparations had high encapsulation yields (Table I). Thus, with $Si(OR_3)_2$-NPc 100% encapsulation yields were observed. These high encapsulation yields of the NPc as compared to the Pc derivatives may reflect the higher lipophilic nature of the former.

Nanoparticles

With Zn-PcS_4, nanoparticles were obtained using different polymerization media and IBCA or EBCA (Table II). IBCA nanoparticle size was generally very small (10- to 20-nm range). A small size was also obtained using SO_2-saturated EBCA and Zn-PcS_4 dissolved in pure water. With more acidic media, the size was markedly larger. In 1% dextran, 10 m*M* phosphate (pH 2.5), nanoparticle size was 378 ± 60 nm.

Binding efficiency of Zn-PcS_4 was constant up to 400 μmol/ml and dropped rapidly above that concentration (Fig. 8). This behavior could be consistent with either covalent binding subsequent to an initiation of the polymerization reaction by Zn-PcS_4 or adsorption of the dye onto the polymer. Should a covalent binding process prevail, the drug in excess of reactive monomer would not be bound. Similarly, in an adsorption process corresponding to a Langmuirian isotherm, when a monomolecular layer of drug has been adsorbed on the polymer the excess remains in solution. The latter mechanism can, however, be considered unlikely because drug added to preformed unloaded nanoparticles was not adsorbed, indicating the low affinity of the drug for the polymer.

Table II. Size and Drug Fixation Capacity of Nanoparticles Prepared in the Presence of Zn-PcS_4

Monomer	Polymerization medium[a]	Size (nm)	Zn-PcS_4 fixation (% initial concentration)
IBCA	A	250	60.8
IBCA	B	21	62.2
IBCA	C	10	64.8
EBCA	A	271	59.7
EBCA	B	10	65.2
EBCA	C	350	55.2
EBCA	D	146	45.3

[a] (A) H_3PO_4 (pH 2.5), 1% dextran 70. (B) Water. (C) HCl (pH 2.5), 5% glucose, 1% dextran. (D) H_3PO_4 (pH 3.5), 1% dextran.

CONCLUSION

This work presents methods that allow efficient encapsulation of phthalocyanines and naphthalocyanines in nanocapsules consisting in an organic core surrounded by a biodegradable polymeric wall. Nanocapsules were easily purified by centrifugation and resuspension. In addition, it was also possible to bind sulfonated phthalocyanines to plain polymeric nanoparticles, which were obtained in a variety of sizes (10- to 380-μm diameter), with different polymers (isobutyl- or ethyl-2-butylcyanoacrylate). Nanoparticles are generally taken up rapidly by reticuloendothelial cells (17). However, small-size nanoparticles (10–20 nm) could prove useful for targeting drugs to parenchymal cells of the liver. Indeed, they should be able to cross the pores (100-μm diameter) of the liver sinusoid endothelial lining sieve-plates (18) and gain access to the Disse space. The different types of phthalocyanine-containing nanospheres could therefore find applications for the photodynamic therapy of malignancies localized in different areas of the body.

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