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Multicomponent polymeric micelles based on polyaspartamide as tunable fluorescent pH-window biosensors

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ABSTRACT

PHEA-PEG$_{5000}$-C$_{16}$ is a polyaspartamide polymer with appended hydrophilic PEG$_{5000}$ functions and hydrophobic n-C$_{16}$ units forming biocompatible micelles with a CAC as low as 1.8×10^{-7} M. The protonation and acidity constants of the polymer's amino and carboxylic groups have been determined by potentiometric titrations at five different concentrations higher than CAC, finding concentration-independent values. Viscosity and polarity of the micellar core have been investigated by means of fluorescent probes, finding local values comparable to those of pure toluene and to the core of sodium dodecyl sulphate micelles, independently on the protonation degree of the polymer. The fluorophore pyrene, the lipophilic N,N′-dimethyl-N″-dodecylamine and 2-dodecylpyridine self-assemble in the hydrophobic core of PHEA-PEG$_{5000}$-C$_{16}$ micelles originating a micellar device that behaves as a rare "off-on-off" fluorescence sensor for pH windows, with no interference by the amino and carboxylic functions of the polymer. The "on" state of the sensor includes the physiological 6–8 pH interval, and can be finely shifted in both directions of the pH axis by comicellization of charged cosurfactants. Dialysis experiments demonstrate that the micellar device exibits an efficient retention ability of all molecular components, including cosurfactants, thus candidating for in vivo use.

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1. Introduction

Due to its relevance for cell biology, the proton concentration was one of the first in vivo parameters to be monitored by fluorescent probes: pH influences processes like cell metabolism and growth, ionic current flow through membrane channels, solute movement on membrane transporter proteins, vectorial water movement, general ion homeostasis (Fliegel, 2005; Hunte et al., 2005) and, in the case of muscle, cellular contractility (Wakabayashi et al., 2006). On the other hand, pH is locally modified by acid-generating processes such as the metabolism of anomalous entities like tumours or bacterial biofilms (Drummond et al., 2000; Murray et al., 2005).

At a closer view, it appears crucial that the pH value for a healthy cell metabolism and for any cell-related process is comprised *inside an interval*, because both lower and higher pH values may have noxious effects. In many cases this interval is the so-called physiological pH range, i.e. 6.8–7.4. Molecular fluorescent sensors capable of signalling if the pH is inside or out a given interval, i.e. with a window-shaped response, are very rare (de Silva et al., 1996, 1997, 2002; Fabbrizzi et al., 1998; Gunnlaugsson, 2001) and new examples must be welcome, in particular if they have biocompatible structures, that may allow an in vivo use.

Recently, micellar fluorescent sensors have been described, in which lipophilic molecular components self-assemble inside micelles in aqueous solutions (Pallavicini et al., 2009a). These micellar devices are capable of efficiently and selectively measuring physical molecular properties (Chirico et al., 2008) and signalling metal cations (Diaz-Fernandez et al., 2004; Mancin et al., 2009; Grandini et al., 1999). The self-assembling micellar approach has allowed some of us to obtain also fluorescent sensors for pH windows, both of the "on-off-on" (Denat et al., 2010) and of the "off-on-off" type (Diaz-Fernandez et al., 2006). The rationale of these micellar sensors is pictorially described in Scheme 1. The "off-on-off" type pH-window sensors were obtained by self-assembling in TritonX-100 micelles a lipophilic fluorophore, a lipophilized pyridine and a lipophilized tertiary amine: on increasing pH the "off-on" and "on-off" transitions are obtained with the deprotonation of pyridinium and with the protonation of amine, respectively, as both a protonated pyridine and a free tertiary amine are well established, efficient quenchers of fluorescence. Accordingly, the "on" window has a

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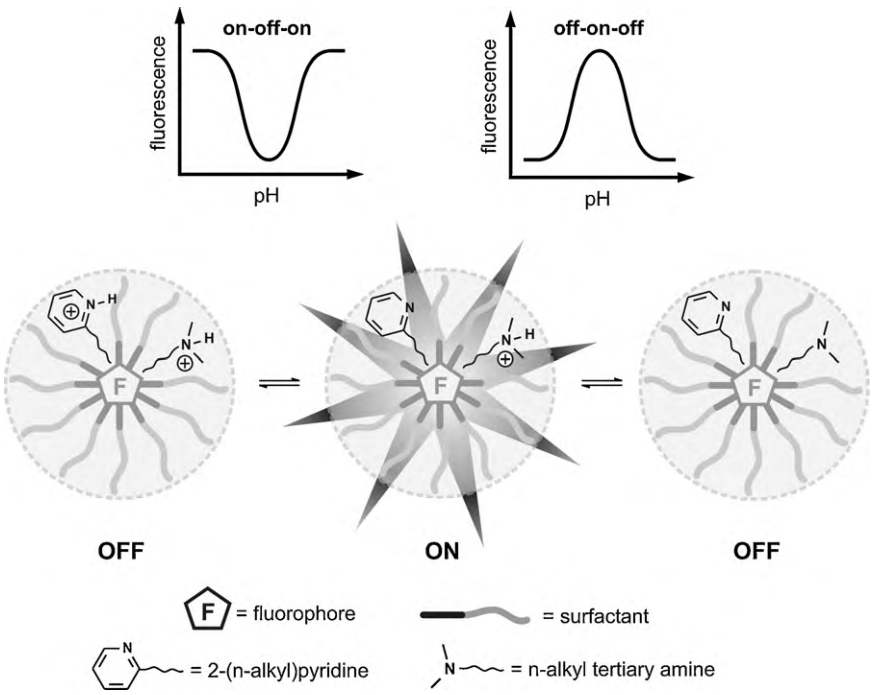

Scheme 1. Top: ideal profile of I_f vs pH for window-shaped pH sensors, both of the "on-off-on" and of the "off-on-off" type. Bottom: working scheme for a fluorescent micellar sensor for pH windows using pyridine and tertiary amines as fluorescence switches.

half-height width dictated by the pyridinium and ammonium pK_a values.

Although efficient and versatile, micellar devices based on low molecular weight surfactants have a huge drawback: they cannot be used in vivo. Low molecular weight surfactants have high critical micellar concentrations (e.g. cmc = 2×10^{-4} M for TritonX-100): in vivo dilution would disrupt the device and release molecular components that may be toxic. On the other hand, if the surfactant would be kept over cmc it would be sufficiently concentrated to seriously damage living organisms. In the perspective of preparing new and biocompatible micellar fluorescent sensors for pH windows, we decided to step to polymeric micelles, that may act more advantageously as the containers for self-assembling the molecular components (Torchilin, 2007). Polymeric micelles are colloidal systems based on amphiphilic copolymers, which are able to self-assemble to micelle-like aggregates in aqueous media. Thanks to their kinetic stability and low CAC (critical aggregation concentration, 10^{-6}–10^{-8} M), these structures are stable at the in vivo dilution. Moreover, in many cases they are biocompatible and do not display cytotoxicity. PHEA-based polymeric micelles (PHEA = poly(hydroxyethylaspartamide)) have been already studied as drug delivery systems. They are capable to improve drugs solubility in water, they are chemically stable and they demonstrated to be biocompatible. In particular, they do not display cytotoxicity in the absence of loaded drugs in a wide range of concentration, as it has been proved by cell viability tests on murine myeloid leukaemia NFS-60 cells (Cavallaro et al., 2003), on human breast cancer MCF-7 cells (Cavallaro et al., 2004) and on colon carcinoma cells (Caco-2), human bronchial epithelial cells (16-HBE), human chronic myelogenous leukaemia cells (K-562) and human-derm fibroblasts (HuDe) (Craparo et al., 2009). Moreover in vivo experiments did not show any acute toxicity effects on mice (Caliceti et al., 2001).

However, it has never been examined the possibility to include in this kind of micelles different molecular species and to allow their interaction to produce new functions, i.e. their use as containers for multicomponent molecular devices. This use is not obvious, if we consider their polymeric, multi-branched nature, that may be an obstacle for intramicellar interactions and communication.

The aim of this work is to demonstrate that polymeric micelles based on the PHEA-PEG_{5000}-C_{16} amphiphilic graft copolymer (Cavallaro et al., 2003, 2006; Craparo et al., 2009) are capable to include multiple molecular components and to promote their intramicellar communication, in order to obtain a new example of the rare category of fluorescent sensors for pH window. As we wanted to work on a device that has to span large pH intervals, we first determined the acid–base equilibria involving the micelles of PHEA-PEG_{5000}-C_{16} alone, see Scheme 2.

Then we self-assembled the molecular components for the fluorescent pH-window sensor in the PHEA-PEG_{5000}-C_{16} micelles. The obtained sensor is of the "off-on-off" type, and the position of the "on" window contains the physiological pH interval. We also demonstrated that these micelles can include additional low molecular weight positively and negatively charged cosurfactants, whose role is to electrostatically shift the transitions between the "on" and "off" states, i.e. to finely tune the position of the "on" window along the pH axis. The formula of the PHEA-PEG_{5000}-C_{16} copolymer and of the molecular components used to obtain the "off-on-off" sensor and tot shift the position of the "on" window are illustrated in Scheme 3.

2. Experimental

2.1. Materials

N,N-Dimethylformamide (DMF), D_2O (isotopic purity 99.9%), N,N′-dimethyl-N″-dodecylamine (**DDA**) were purchased from Sigma–Aldrich (Italy). O-(2-Aminoethyl)-O′-methyl polyethylene glycol 5000 Da (PEG_{5000}-NH_2) (<0.17 mmol NH_2/g), hexadecylalkylamine (C_{16}), ethanol, ethanolamine, pyrene were from Fluka (Italy). 2-Dodecyl-pyridine (**DP**) was prepared according to a described procedure (Brody et al., 1943). Sodium dodecyl sulphate (SDS), cetyl trimetylammonium bromide (CTAB) were purchased from Aldrich and used without further purification. Polysuccin-

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