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Novel micellar form of poplar propolis with high cytotoxic activity†

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A novel micellar form of propolis based on a biocompatible poly(ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide) *block* copolymer ($PEO_{26}PPO_{40}PEO_{26}$) is developed. Beneficially, all biologically active lipophilic constituents of propolis are solubilized with the aid of polymeric micelles in aqueous media. The micellar form of the propolis exhibits high *in vitro* cytotoxicity at relatively low concentrations.

Natural products possessing pharmacological or biological activity have been among the most intensively studied substances for the design and discovery of new drug formulations.[1] They have historically been the most productive source of active compounds in the development of new medicines. For instance, 48.6% of the 175 anti-cancer agents approved by the U.S. Food and Drug Administration from 1940 until 2012 are natural products or directly derived from natural products.[2]

Propolis, also called "bee glue", is a resinous hive product manufactured by bees. The honeybees collect lipophilic resinous material from different parts of living plants, mix it with wax and use it in the hive as a building material and as a protective material against microbial infections.[3] Propolis contains secondary plant metabolites which play protective role in the source plants. The action of propolis against infections has been recognized by human beings millennia ago and it has been widely used in traditional medicine of different cultures for treatment of wounds and burns, sore throat, stomach ulcer, *etc.*[4] Modern science has confirmed the antimicrobial and antiviral action of propolis, and has discovered numerous other beneficial pharmacological properties of bee glue: immunomodulating, antiinflammatory, antioxidant, antiulcer, antidiabetic, antiobesity properties and many others.[5] In particular, propolis might be considered as an important source of natural bioactive compounds for cancer treatment, due to its potent apoptotic effect leading to death of cancer cells. Several *in vitro* studies have shown a remarkable activity of the basic compounds of propolis alcoholic extracts against different tumor cells.[6] However, the poor solubility of the active lipophilic secondary plant metabolites in aqueous/biological media seems to reduce their bioavailability, which is one the main obstacle for application of propolis in cancer therapy. Indeed, the *in vivo* studies reported so far are based on water-soluble fractions of propolis which can be applied both orally and parenterally.[7]

Block copolymer aggregates like micelles, vesicles, *etc.* have been intensively studied for development of chemotherapeutical formulations due to their ability to solubilize different anticancer compounds.[8] Beside the huge number of copolymers, poly(ethylene oxide)-*block*-poly(propylene oxide)-*block*-poly(ethylene oxide) triblock copolymers are among the most attractive copolymers for preparation of drug and gene delivery systems.[9] They are commercial biocompatible copolymers available in a broad range of compositions and molar masses, and spontaneously form micelles or other aggregates in water above a critical concentration and temperature. PEO–PPO–PEO copolymer micelles combine the good resistance of PEO shell against protein adsorption and cellular adhesion (stealth effect) with the ability of the temperature-responsive PPO core to solubilize poorly water soluble drugs. In addition, the available terminal hydroxyl groups allow for attachment of receptor-specific ligands used for the so-called active targeting of carriers. PEO–PPO–PEO micelles have demonstrated their potential for encapsulation and sustained release of various natural anticancer compounds like paclitaxel,[10] curcumin,[11] silybin,[12] *etc.* *In vivo* experiments revealed a significant improvement of the pharmacokinetic parameters after

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administration of micellar formulations and increased time of systemic circulation.

The present communication reports on novel delivery system based on $PEO_{26}PPO_{40}PEO_{26}$ copolymer micelles (Pluronic P85, BASF) which are able to solubilize all biologically active components from the ethanol extract of poplar propolis (*Populus nigra* L.). To the best of our knowledge, this is the first contribution focused on the encapsulation of the full spectrum of lipophilic propolis compounds in block copolymer micelles. This method paves the way for parenteral administration of propolis in aqueous media and cancer-related applications. The encapsulation of propolis within the polymeric micelles was achieved by self-assembly of $PEO_{26}PPO_{40}PEO_{26}$ triblock copolymer in ethanol/water media in the presence of propolis and subsequent evaporation of the organic solvent. Firstly, poplar propolis was extracted with ethanol/water (70/30 v/v) mixture for 24 h at room temperature. The solution was filtered through filter paper and the concentration of dissolved propolis (4.9 mg mL^{-1} dry extract), as well as the concentration of the bioactive constituents (total phenolics 3.2 mg mL^{-1}, total flavones/flavonols 0.8 mg mL^{-1} and total flavanones/dihydroflavonols 0.6 mg mL^{-1}) was determined spectrophotometrically. The chemical composition was determined by GC-MS analysis of the dried extract after silylation (Table S1 – ESI†). Then, given amount of $PEO_{26}PPO_{40}PEO_{26}$, dissolved in ethanol/water (70/30 v/v) mixture, was added to the propolis solution. Finally, ethanol was removed from the system by rotary evaporator, giving a transparent yellowish colloidal aqueous solution (Fig. 1, left). The obtained colloidal solution retained its essential characteristics (transparency, colour, stability) upon storage at temperature of 20–25 °C for more than one year. In a control experiment, the same procedure was repeated without use of copolymer. In this case, a turbid suspension was formed (Fig. 1, right), which tended to precipitate after 2 days storage. The solubilization ability of $PEO_{26}PPO_{40}PEO_{26}$ as a function of copolymer to propolis weight ratio and concentration was studied in detail. Dynamic light scattering (DLS) measurements of samples containing 1 mg mL^{-1} propolis revealed formation of nano-sized aggregates with monomodal particle size distribution, and an apparent hydrodynamic diameter $D_h = 97$ nm and dispersity 0.21 (Fig. S1†) at a copolymer to propolis wt ratio 2.

At lower copolymer content only turbid and unstable colloid systems were formed, while at fivefold excess of copolymer the hydrodynamic diameter of micelles obtained was reduced to approximately 20 nm (Fig. 2). In addition, the increase of propolis concentration up to 20 mg mL^{-1} did not alter the tendency for formation of nano-sized micelles within the above-mentioned experimental conditions. For instance, at propolis concentration of 20 mg mL^{-1}, the micellar aggregates formed at copolymer to propolis wt ratio from 3 to 10 exhibited apparent D_h approximately 20 nm. Transmission electron microscopy (TEM) analysis confirmed the formation of spherical micelles with diameter in the range of 10–20 nm (Fig. 3). One can suggest that the high contrast of the objects is due to the encapsulated propolis. Further on, the study was extended to evaluation the capacity of other representatives of "Pluronic family" to solubilize poplar propolis in water. Following the same preparation protocol, $PEO_{20}PPO_{70}PEO_{20}$ (P123), $PEO_{19}PPO_{29}PEO_{19}$ (P65), $PEO_{43}PPO_{15}PEO_{43}$ (F38) and $PEO_{75}PPO_{30}PEO_{75}$ (F68) copolymers were tested. The copolymers with a higher PEO content, F38 and F68, was unable to form micellar solutions (D_h > 200 nm) even at a copolymer to propolis wt ratio 10, while P123 and P65 formed stable micellar solutions above wt ratio 2 and 10, respectively. Obviously, to encapsulate propolis in copolymer micelles one need to select a copolymer with proper characteristics, which allow to achieve the combination of good colloidal stability of the micellar system and high loading capacity. However, further studies are needed to understand the role of copolymer composition and molar mass for effective encapsulation of propolis in PEO–PPO–PEO micelles.

In vitro release of propolis from $PEO_{26}PPO_{40}PEO_{26}$ micellar formulation in phosphate buffer (pH 7.4) was investigated by UV-vis spectroscopy using the dialysis method. The intensity of adsorption spectrum with maximum at 317 nm (Fig. S2†) was taken into account for the calculation of released propolis for given time interval. The results revealed a sustained release of propolis in the phosphate buffer with a weak burst effect. In fact, approximately 100% of propolis was released from the micelles for 52 hours (Fig. S3†). The achievement of sustained



Fig. 1 Digital micrograph of colloidal aqueous solution of $PEO_{26}PPO_{40}PEO_{26}$/poplar propolis (left) and aqueous suspension of poplar propolis (right). Concentration of propolis 2 mg mL^{-1}; copolymer to propolis wt ratio 3.

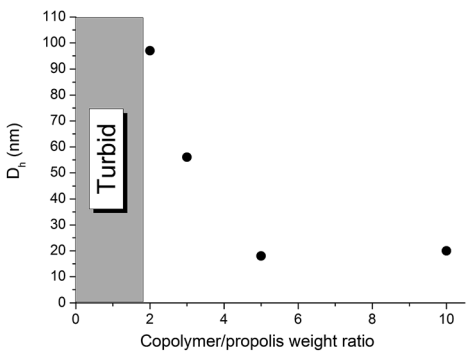


Fig. 2 Effect of copolymer to propolis wt ratio on the preparation of stable colloidal aqueous solution. Concentration of propolis = 1 mg mL^{-1}.

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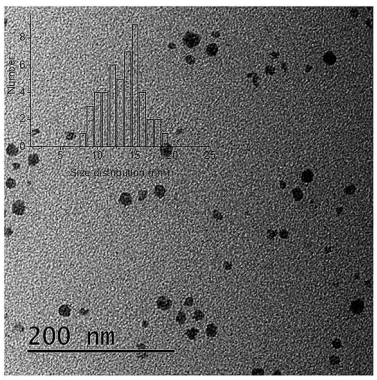


Fig. 3 TEM micrograph of poplar propolis encapsulated in PEO_{26}-$PPO_{40}PEO_{26}$ micelles. The sample is prepared from micellar solution formed at copolymer to propolis wt ratio 3 and concentration of propolis 2 mg mL^{-1}. The inset shows the size distribution of micelles.

release is probably due to the hydrophobic interactions between propolis and PPO blocks.

In vitro cytotoxic activity of free propolis, propolis loaded in $PEO_{26}PPO_{40}PEO_{26}$ micelles and blank $PEO_{26}PPO_{40}PEO_{26}$ micelles was evaluated by MTT test on HL-60, REH and HEP-G2 cells (Fig. 4). The results showed that the blank micelles had no effect on the cells. Aqueous systems of free propolis and micellar propolis demonstrated dose dependent cytotoxicity on leukemic cells. Importantly, the cytotoxic activity of micellar propolis was remarkably more pronounced on the selected cell lines as compared to the free propolis. In the case of HL-60 cells, micellar propolis reduced cell viability with approximately 65% even at the lowest concentration (10 μg mL^{-1}), whereas at the same concentration the free propolis was not active. Similar tendency was observed on REH cell line, where at 30 μg mL^{-1} micellar propolis reduced the viability with 35% *vs.* 18% for the free propolis. Regarding hepatocellular carcinoma cell line, the free propolis was not active, whereas the micellar propolis showed activity in the range of 30–50 μg mL^{-1}. These data suggested that the micellar formulation of propolis enabled very high activity even at low concentration, which was not observed for the free propolis.

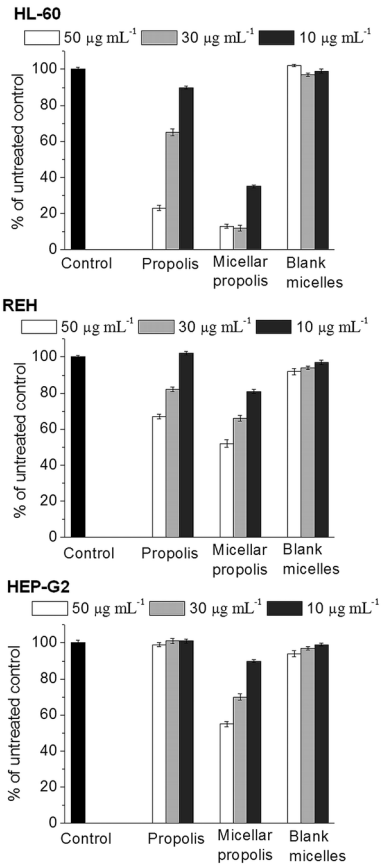


Fig. 4 *In vitro* cytotoxicity of free propolis, micellar propolis and blank micelles on HL-60, REH and HEP-G2 cells. Mean ± SD ($n = 6$).

Conclusions

Efficient and facile approach for preparation of aqueous colloidal solution of poplar propolis by encapsulation of the natural products within $PEO_{26}PPO_{40}PEO_{26}$ micelles was developed. Stable system was obtained at a relatively low copolymer to propolis weight ratio. The nanometer size, fairly high cytotoxic activity on cancer cells and the sustained release profile makes micellar propolis a promising candidate for cancer-treatment applications. We suggest that the described approach is general, and stable aqueous colloidal solution of propolis can be prepared by using other amphiphilic copolymers. This could boost an extensive research in the field of propolis based nanomedicine.

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