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L-carnitine Derived Zwitterionic Betaine Materials

Wei Wang, Jianhai Yang, Ershuai Zhang, Yang Lu, and Zhiqiang Cao

Department of Chemical Engineering and Materials Science, Wayne State University, Detroit, Michigan, 48202, USA

Abstract

An L-carnitine derived zwitterionic betaine material was developed. The synthesized L-carnitineMA was the first zwitterionic betaine monomer from natural feedstocks. In the models of Surface coating and hydrogel, the L-carnitineMA material was able to achieve superior anti-fouling performance as good as state-of-the-art zwitterionic betaine materials

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The L-carnitineMA was the first zwitterionic betaine monomer from natural feedstocks and achieve superior anti-fouling performance.

Zwitterionic betaine materials, such as poly-carboxybetaine (PCB), poly-sulfobetaine (PSB) and poly-phosphorylcholine (PPC), have been intensely studied for applications in the fields of biomedical devices¹ and marine coating industry² as an effective anti-fouling strategy to avoid protein binding and microorganism adhesion. Currently, most of these betaine polymers or monomers are synthesized from petrochemical derivatives³. As global fossil fuel dwindles, there is strong interest, though limited research⁴, in producing these materials from the natural feedstock. Here we report the development of a single-step synthesis protocol to obtain a novel zwitterionic carboxybetaine monomer using L-carnitine as a natural product starting material. We further show that the obtained L-carnitineMA monomer can be used to prepare polymer surface coatings or bulk hydrogel materials with superior antifouling performance, comparable to the state-of-the-art carboxybetaine

Correspondence to: Zhiqiang Cao.

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Conflict of interests

The authors declared no conflicts of interests

materials such as poly-carboxybetaine methacrylate (PCBMA)⁵. To the best of our knowledge, the synthesized L-carnitineMA monomer is the first ever reported betaine monomer derived from natural feedstocks.

There is limited research in developing zwitterionic betaine materials from natural products. It has been reported that the hydroxyl groups of a natural polysaccharide such as dextran could be modified with carboxybetaine groups⁴; however, the modification efficacy was low due to steric effect and potentially compromised the antifouling property compared with polymer materials prepared from monomers with betaine moieties⁶. Therefore, we sought to develop novel zwitterionic betaine small molecule monomers that could be further polymerized for a variety of applications. We focused on L-carnitine as the natural starting material because: (1) L-carnitine can be obtained in large scale. As a natural derivative of the amino acid, it is routinely produced by either extracting from the natural product (usually from beef)⁷ or through enzyme-catalyzed synthesis⁸. (2) L-carnitine has a good safety profile. L-carnitine product was approved for treatment of Dialysis-related Carnitine Deficiency (DCD) in as early as the 80s⁹. So far, it has been globally approved as a non-toxic food and drug additive in soft drinks, biscuits and dried milk, as well as various health products¹⁰. (3) The structure of L-carnitine offers a zwitterionic carboxybetaine moiety and a hydroxyl group for chemical reaction to form a monomer. Here we synthesized zwitterionic betaine monomer (L-carnitineMA) through a single-step reaction by reacting L-carnitine with acryloyl chloride. Feasible reaction conditions and purification procedures were explored, and the obtained L-carnitineMA was confirmed with nuclear magnetic resonance (NMR) and mass spectroscopy. We prepared polymer coatings by atom-transfer radical-polymerization (ATRP) and hydrogels through crosslinking, and verified the anti-fouling performance to be comparable to the state-of-the-art carboxybetaine materials (CBMA) using surface plasmon resonance (SPR) sensor and enzyme-linked immunosorbent assay (ELISA) test to quantify protein adsorption, and assays to evaluate bacterial adhesion.

The synthesis of the L-carnitineMA was designed using a one-step reaction (Fig. 1). Briefly, 1.97g L-carnitine hydrochloride (10 mmol) and 100 mg hydroquinone were added into 25 ml anhydrous dimethylformamide (DMF) in a flask purged with nitrogen. The mixture was heated to 40 °C and stirred for 10 mins. Then 2.43 ml acryloyl chloride (30 mmol) was dropped into the solution and the temperature was raised to 80 °C to react for 3 h. During the reaction, the flask was connected to a tube that leads to a liquid-seal bottle filled with triethylamine (TEA) to absorb excessive hydrogen chloride (HCl) from the reaction. The starting material L-carnitine hydrochloride gradually dissolved and the solution turned clear and brown.

It is critical to select the right solvent for the one-step reaction to obtain L-carnitineMA. Acryloyl chloride has to be dissolved in a non-protic solvent to preserve its reactivity. L-carnitine on the other hand is hydrophilic and can hardly be dissolved in most non-protic solvents such as acetonitrile and DCM (Table S1). By increasing the temperature, L-carnitine hydrochloride can be partially dissolved in DMF and dimethyl sulfoxide (DMSO). Since DMSO drastically reacted with acryloyl chloride through Swern-oxidation¹¹, DMF was chosen as the solvent.

The reaction temperature was chosen at 80 °C. This is because that L-carnitine hydrochloride can be partially dissolved in solvent DMF at this temperature to precipitate the reaction. In addition, maintaining high temperature (40 °C or above) is critical since hydroquinone was oxidized to benzoquinone which is a polymerization inhibitor and does not react with acryloyl chloride leading to side products. Our data show at room temperature hydroquinone reacted with acryloyl chloride significantly (calculated Mw $C_{12}H_{10}O_4+H$ was 219.7, confirmed by mass spectroscopy of an m/z of 220.1).

In a typical reaction between the hydroxy group and acryloyl chloride, TEA is frequently added to the reaction mixture as the deacid reagent to increase the reaction efficiency. However, TEA cannot be used in our system because (1) it turned the L-carnitine from hydrochloride form to inner-salt form, which was totally insoluble in DMF even at 80 °C (Table S1); (2) at high temperature, the acryloyl chloride reacted with TEA significantly and produced side products that complicated the purification procedure¹².

After the single-step reaction, the rough product was purified as follows. The majority of hydrogen chloride generated and the excessive amount of acryloyl chloride was removed under vacuum at room temperature by stirring the reaction solution. Then the resulting mixture was placed at -20 °C overnight. Unreacted L-carnitine hydrochloride was precipitated and removed by filtration. The resulting solution was further treated by anhydrous TEA where the L-carnitineMA product, triethylamine hydrochloride and certain colored impurities were precipitated. The precipitate was washed with dichloromethane (DCM) to remove triethylamine hydrochloride and was vacuum-dried. To remove the colored impurity, the obtained product was dissolved in anhydrous methanol stirred with activated charcoal for 2 h at 40 °C. The supernatant was obtained by centrifugation, precipitated in diethyl ether and dried in vacuum. L-carnitineMA in the form of light-yellow powder was obtained at the optimized yield of ~43 %.

The structure of L-carnitineMA monomer was confirmed with NMR (Fig. 2). Compared with unreacted L-carnitine (Fig. S1), L-carnitineMA showed three new NMR peaks (a, b and c) corresponding to the three hydrogens on the double bond structure. The area ratio of peak d (9 hydrogens from the quaternary ammonium structure) and peak b (1 hydrogen from the double bond structure) was 8.87, indicating no polymerization occurred during the monomer synthesis. The mass spectrometer further confirmed the success of synthesis by giving an m/z of 216.2 for the L-carnitineMA (calculated Mw for L-carnitineMA+H was 216.4).

To evaluate the anti-fouling performance of the L-carnitineMA based materials, we prepared a polymer brush coating on the gold substrate as a model system. We first immersed the SPR gold chip in an ethanol solution containing 1 mM initiator ω -mercaptoundecyl bromoisobutyrate (synthesized as previously reported¹³) overnight. The initiator was immobilized on gold surface through Au-S bond, and loosely bound initiator was removed by rinsing the surface with pure ethanol and tetrahydrofuran (THF) (detailed procedure see ESI). The immobilized initiator was used to initiate the ATRP of L-carnitineMA monomer in presence of Copper (I) bromide (CuBr) and 2,2'-bipyridine (BPY) (detailed procedure see ESI). The obtained L-carnitineMA polymer brush surface was washed several times with fresh DI water, and its thickness was measured by surface ellipsometry.

We generally found the reaction temperature (room temperature or 40 °C) and the reaction time (3 h or longer) did not affect the coating thickness significantly (differences less than 2%). The reaction solvent or mixed solvent, however, had a profound impact on the coating thickness (Table S2). It appeared that the use of methanol in the mixed solvent decreased the obtained polymer brush thickness. Water/DMF system was more suitable to grow L-carnitineMA polymer films, and at 2:1 water/DMF volume ratio, the film thickness reached 23.0 ± 3.8 nm (average $\pm$ standard deviation, $n=3$). This thickness is comparable to 26.6 ± 0.5 nm of PCBMA brush coating, which shows superior anti-fouling performance¹⁴.

The morphology of L-carnitineMA polymer brush surface was significantly different from bare gold surface as visualized by the tapping mode of atomic force microscope (AFM) (Fig. 3(a)). The thickness of L-carnitineMA polymer brush surface was also measured by AFM using a reported method¹⁵. A shallow scratch was made on the L-carnitineMA polymer brush surface using a sharp lancet. By measuring the depth of the scratch, the polymer brush thickness was calculated (Fig. S2) to be 20.5 ± 4.6 nm (average $\pm$ standard deviation, $n=4$), in agreement with the thickness value measured by surface ellipsometry.

The obtained L-carnitineMA polymer brush coated gold chip (~20 nm thick) was measured for anti-fouling capability by surface plasmon resonance (SPR) sensor. The negatively-charged fibrinogen and the positively-charged lysosome were utilized in the test. Protein solutions of 1.0 mg/mL in PBS (0.15 M, pH 7.4) were injected over the tested surfaces for 10 min followed by PBS buffer injection for 10 min to remove loosely bound proteins. The wavelength changes between the baselines before the protein injection and after the removing buffer injection was used to calculate non-specific protein adsorption on the surface. Undetectable adsorption (< 0.3 ng/cm²) of both fibrinogen and lysosome was observed (Fig. 3(b)). The results suggested that the obtained L-carnitineMA polymer coated surface was able to achieve “super-low-fouling” against protein binding that is comparable to PCBMA (< 0.3 ng/cm²) coatings¹⁴. Super-low fouling is defined by being able to resist protein adsorption down to < 5 ng/cm², which is desirable to inhibit platelet adhesion for blood compatibility¹⁶. As a reference, a monolayer of protein binding resulted in a sensor response of 100–500 ng/cm²¹⁴.

The L-carnitineMA polymer brush coating was further challenged in a microorganism adhesion test using *E. coli* K12 as the model bacterium. The bare gold surface, state-of-the-art PCBMA polymer brush coating (positive control) and L-carnitineMA polymer brush coating were placed in the bacteria suspension (10^8 cells/ml) for 0.5 h, followed by a gentle wash with PBS to remove loosely bound bacteria. The samples were then fixed, dehydrated, vacuumed and coated with nano-gold layer followed by SEM imaging (detailed procedure see ESI). SEM images (Fig. 4) showed that the L-carnitineMA polymer brush surface greatly reduced the bacteria adhesion (0.28 ± 0.03 bacteria per 100 μm^2 (average $\pm$ standard deviation, $n=6$)), as compared to the uncoated bare gold surface (11.4 ± 2.2 bacteria per 100 μm^2). The bacteria adhesion on PCBMA was 0.32 ± 0.05 bacteria per 100 μm^2 , and there was no significant difference between the PCBMA coated surface and L-carnitineMA polymer coated surface in bacteria adhesion density at $p < 0.0001$ (Fig. 4(d)). Results indicate that the L-carnitineMA polymer has the same superior anti-fouling capability against bacteria adhesion as the state-of-the-art zwitterionic betaine polymer.

In addition to prepare brush coatings from L-carnitineMA, we further obtained hydrogel network (Fig. 5(b)) using L-carnitineMA monomer and commercial N,N'-Methylenebisacrylamide (MBAA) crosslinker through UV initiation (see ESI). The anti-fouling property of the L-carnitineMA derived hydrogel in resisting protein binding was evaluated by enzyme-linked immunosorbent assay (ELISA) using the human fibrinogen as the model protein (detailed procedure see ESI). Results indicated that the L-carnitineMA hydrogel showed better anti-fouling property compared to poly-(Hydroxyethyl)methacrylate (PHEMA) hydrogel (negative control), and was able to achieve a similar anti-fouling level as the commonly used PCBMA hydrogel (Fig. 5(a))¹⁷.

Conclusion

In summary, we demonstrated, for the first time, the synthesis of zwitterionic betaine monomer, L-carnitineMA, through simple-step reaction from L-carnitine, a natural feedstock starting material. We further demonstrated that surface coatings and hydrogel materials made from L-carnitineMA achieved superior anti-fouling performance as good as state-of-the-art zwitterionic betaine materials. As an alternative to current carboxybetaine monomers, L-carnitineMA is expected to be potentially applied in a broad range of applications (e.g., antifouling and antimicrobial coating¹⁸, implantation¹⁹, drug delivery²⁰, and tissue engineering²¹), where current zwitterionic betaine materials play a decisive role.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Synthesis route of L-carnitineMA

Fig. 2.
 ^1H NMR characterization of L-carnitineMA

Fig. 3.

(a) AFM image of bare gold surface and L-carnitineMA polymer brush surface, scale bar = 10 nm, and (b) Adsorption of 1 mg/mL fibrinogen and lysozyme in PBS buffer on L-carnitineMA polymer coated gold surface as determined by SPR

Fig. 4.

E. coli adhesion test on (a) bare gold surface, (b) PCBMA polymer brush coating and (c) L-carnitineMA polymer brush coating, and (d) calculated bacteria adhesion density. Statistical analysis: one-way ANOVA with Bonferroni multi-comparison. ***: $p < 0.0001$. n.s.: no significant differences, $p > 0.5$. Scale bar = 10 μm.

Fig. 5. (a) protein binding test on HEMA, poly-L-carnitineMA and PCBMA hydrogel, and (b) optical image of poly-L-carnitineMA hydrogel